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STUDIES ON VIBRIONIC INFECTION OF SHEEP

I. THE AGGLUTINATION TEST AS A MEANS OF DIAGNOSIS

II. THE ANTIGENIC RELATIONSHIP BETWEEN STRAINS OF *V. foetus*

By

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I. THE AGGLUTINATION TEST AS A MEANS OF DIAGNOSIS

Although the diagnosis of vibrionic abortion in sheep is sometimes attempted by using the agglutination test, very little information exists upon the significance to be attached to the results. It was therefore decided to determine its value for this purpose and to ascertain the best procedure to adopt.

Since vibrionic abortion amongst sheep was first recognised by M'Fadyean and Stockman (1913) it has been described in many parts of the world both in cattle and in sheep, although the relation between the sheep and cattle diseases has not been determined. M'Fadyean and Stockman found the abortion rate in affected flocks of sheep to be between 5 and 50 per cent. In our experience over several years the abortion rate varies within wide limits, being insignificant in some flocks and in others affecting up to 90 per cent. of the pregnant ewes. Abortion usually occurs late in pregnancy. Ryff (1940), however, observed a few early abortions which is in agreement with our findings. The natural method of spread has not been ascertained with certainty, but M'Fadyean and Stockman produced some evidence that pastural infection is possible, while Buxton (1930) reported a case in which a ram appeared to transmit infection.

Several workers have considered the aetiological status of *V. foetus*. M'Fadyean and Stockman showed that recently isolated cultures were much more virulent than old laboratory strains and that the disease could be reproduced with the organism administered by the intravenous, oral or vaginal routes, although only approximately 50 per cent. of the experimental ewes aborted. Smith (1923), working with *V. foetus* infection in cattle, also found that it could not be regularly reproduced. It is not impossible that some unknown predisposing factors acting in association with *V. foetus* may therefore operate to produce the natural disease.

Vibrio foetus is not easily recovered from infected foetuses, especially if other organisms are present. Diagnosis is sometimes based on demonstrating the organism microscopically in smears of uterine exudate or foetal tissues. M'Fadyean and Stockman used various media for isolation, incubating them under aerobic conditions. Smith and Taylor (1919) observed that the organism grew best in an atmosphere in which the CO_2 tension

was raised. They did not recommend liquid media since they found that the organism developed best in the water of condensation of agar slopes. We, too, have found that the organism is slightly microaerophilic. When an abundant inoculum is used growth occurs in an ordinary atmosphere. We have also noticed that an enrichment of the medium is not essential for propagation of cultures but for primary isolation of the vibrio it is advisable to add serum or blood to the broth.

Although *Vibrio foetus* is usually regarded as actively motile, Smith (1923) recovered non-motile strains from cattle, and we have observed that some strains quickly lose their motility when grown under artificial conditions. Our observations on the morphology of the organism are in agreement with those of M'Fadyean and Stockman, who noted that it takes the form of a spirillum and tends to form granules in old cultures. The optimum temperature for growth is 37° C., but multiplication takes place slowly at room temperature.

AGGLUTINATION TESTS WITH OVINE SERA

Four strains of *V. foetus* were recovered from aborted lambs during the course of outbreaks in widely separated parts of East Anglia. Two (VB and VC) became non-motile after a few sub-cultures but enough flagellar antigen had been prepared from VB to carry out tests with it before it had lost motility. The other strains, VS and VT, remained motile.

Methods.—Antigens were prepared by growing the strains at 37° C. under aerobic conditions for three days in flasks containing a litre of 5 per cent. horse serum broth. The cultures were examined for purity and any showing a granular type of growth were rejected as unsuitable. The final antigen consisted of the centrifuged deposit resuspended in merthiolated saline to an opacity equal to Brown's tube No. 3. The agglutination tests were carried out in twofold serial dilutions starting at 1 : 20 and the final readings were made after 18 hours in the incubator at 37° C.

The results of agglutination tests with blood from normal sheep

Samples of blood were obtained from ten shearlings and ten ewes belonging to a flock of sheep in which the abortion rate had been insignificant over a number of years, and the titres at which they agglutinated strain VS were determined. The samples from one of the ewes and one of the shearlings agglutinated the antigen almost completely at 1 : 20, but in the remainder there was only a trace or no agglutination at this dilution. Buxton (1930) recorded that the normal sheep he examined gave a negative result at 1:10, and Ryff (1940) found that in nine normal sheep there was partial agglutination at 1:12. It would seem that while the sera of most normal sheep may give a completely negative result at a dilution of 1 : 20, an occasional sample gives some agglutination at this level but that such reactions cannot be regarded as significant. Furthermore, as the sera were tested against only one strain it is possible that higher titres would have been obtained if cultures from other outbreaks had been used.

The agglutinating titres of sera in a flock in which vibrionic abortion had been diagnosed and strain VB isolated

M'Fadyean and Stockman (1913) carried out agglutination tests with sera from ewes infected with *V. foetus* and obtained titres ranging from 1 : 25 to 1 : 1,000. Buxton (1930) recorded titres of 1 : 8 to 1 : 16 in four affected sheep, but partial agglutination was noted to a dilution of 1 : 64 in two of them. Ryff (1940) examined ewes from a flock severely affected with vibrionic abortion of which 37 had a titre greater than 1 : 25 ; ten of these, four of which were known to have aborted, agglutinated the organism to 1 : 100.

The agglutinating titres of samples from different groups of sheep in a flock were tested against antigen prepared from the infecting strain VB, which was at that time motile. The samples were obtained two months after the outbreak from ewes which had aborted, normal and barren incontact ewes and normal lambs. The results are shown in Table I. If almost complete clearing (+++)

TABLE I

AGGLUTINATING TITRES OF SHEEP (VB OUTBREAK) AGAINST MOTILE VB ANTIGEN

Sheep number	*Titre against Strain VB (motile)*			
	20	40	80	160
BA1	++	++	+	0
BA2	+++	++	+	+
BA3	+++	++	++	+
BA4	++++	++++	++++	+++
BA5	++++	+++	++	+
BA6	++++	++++	++	+
BA7	++++	+++	+++	+++
BA8	+++	+++	+++	+
BA9	++	0	0	0
BA10	+++	++	++	+
BF1	++	+	0	0
BF2	+	+	0	0
BF3	++	+	+	0
BF4	+++	++	0	0
BF5	++++	+++	++	0
BF6	++++	++	0	0
BF7	+++	++	+	0
BF8	++	0	0	0
BF9	++++	+++	+	0
BF10	++	++	+	0
BL1	+++	++	0	0
BL2	+++	++	0	0
BL3	0	0	0	0
BL4	++++	+++	0	0

BA1 – BA10. Ewes which aborted.
BF1 – BF10. In contact ewes which were barren.
BL1 – BL4. In contact ewes which lambed normally.

Of ten lambs born the previous season and reared apart, one had a titre of 1 : 20 +++, 1 : 40 ++, the rest having no titre whatever.

In all tables ++++ signifies complete, +++ almost complete, ++ appreciable, and + trace agglutination.

at 1 : 40 is regarded as significant, five of the ten ewes which aborted, and two of the ten barren ewes, gave a positive result. Only one of the samples from the lambs born the previous season and reared apart from the main flock agglutinated the organism to this degree (*vide* Table I). None of the titres is, however, high and it is obvious that all the infected animals could not be detected by the agglutination test.

The persistence of agglutinins in the blood of infected ewes. Blood samples were taken from ten ewes in another flock 14, 60, 120 and 210 days after abortion and the titres determined against motile strain VS which was isolated from the outbreak. The results, depicted in Table II, show that the titres fell rather sharply 14 to 60 days after abortion. Taking 1 : 40 as a diagnostic titre, the infected ewes, as recorded at the tests, number seven, three, three and three, respectively. A further point of interest manifested in the table is that some sera possess a higher titre at a later bleeding than at the preceding one; this is especially noteworthy of S6 at the third bleeding and S8 at the fourth bleeding. This result would suggest that infection or re-infection may occur between gestations.

TABLE II

THE PERSISTENCE OF AGGLUTININS IN INFECTED EWES AGAINST STRAINS VS (MOTILE) ISOLATED FROM THE FLOCK

Sheep number	*Days after abortion*			
	14	60	120	210
S1	40	20	20	20
S2	40	20	0	20
S3	40	80	40	20
S4	20	0	0	0
S5	40	20	0	0
S6	20	0	80	40
S7	320	80	40	80
S8	160	40	0	40
S9	160	0	0	0
S10	20	20	0	0

Titre recorded is last tube showing almost complete agglutination (+++).

A comparison of the titres of infected ewes in two flocks against homologous and heterologous strains. The agglutinating titres of seven sera taken from ewes one to five days after abortion and tested against the motile strain VT isolated from the flock and VS isolated from another outbreak are shown in Table III. If complete agglutination at 1 : 40 is taken as a positive titre, all seven would be diagnosed as positive with strain VT as against three with strain VS. It was not possible to determine the end point of some of these sera against VT as the amount of this antigen available was limited. A similar test was carried out with the ten sera obtained 14 days after abortion from the outbreak yielding strain VS, against this strain and against

TABLE III

TITRES OF INFECTED SHEEP (VT OUTBREAK) AGAINST MOTILE STRAINS VT AND VS

Sheep number	*Titre against VT (motile)*					*Titre against VS (motile)*				
	20	40	80	160	320	20	40	80	160	320
T1	++++	++++	++++	+++	++	++++	+++	++	+	—
T2	++++	++++	+++	++	—	++	+	—	—	—
T3	++++	++++	++++	++	—	—	—	—	—	—
T4	++++	++++	++++	++++	+++	++	+	—	—	—
T5	++++	++++	++++	+++	++	++++	+++	++	—	—
T6	++++	++++	+++	++	—	++++	+++	++	+	—
T7	++++	++++	++++	++++	+++	+++	++	—	—	—

TABLE IV

TITRES OF SHEEP (VS OUTBREAK) AGAINST VS (MOTILE) AND VB (NON-MOTILE)

Sheep number	*Antigen*									
	VS					*VB*				
	20	40	80	160	320	20	40	80	160	320
S1	++++	++++	+	—	—	++	+	—	—	—
S2	++++	+++	+	—	—	++	+	—	—	—
S3	+++	+++	++	—	—	+++	+	+	—	—
S4	+++	++	—	—	—	+++	++	—	—	—
S5	++++	+++	++	—	—	++	+	+	—	—
S6	+++	++	++	—	—	++++	+++	++	—	—
S7	++++	++++	++++	++++	++++	++++	+++	++	—	—
S8	++++	++++	++++	+++	+	+++	+	—	—	—
S9	++++	++++	+++	+++	+	+++	++	++	—	—
S10	+++	++	+	—	—	++++	+++	+	—	—

non-motile strain VB. The results shown in Table IV indicate that, using the same diagnostic titre, seven would be diagnosed as positive with VS as against three with VB. It would appear, therefore, that sera of infected sheep agglutinate a strain isolated from the same flock to a higher titre than other strains and the extent to which strains not isolated from the flock are agglutinated would not appear to depend on their motility.

DISCUSSION

The diagnosis of vibrionic abortion cannot be considered without reference to the aetiological significance of *V. foetus*. In this connection we are unfortunately not in a position to give an opinion based on experimental data, but the published results of other workers and general experience of the disease in the field do not preclude the possibility that factors other than *V. foetus* may assist in the development of the natural disease. Until it is definitely determined whether or not vibrionic abortion is a specific entity it would seem advisable to regard it as one, but every possible con-

sideration should be given to the aetiological significance of other factors such as malnutrition, dietetic errors, etc., which may exist at the time.

The results show that if serum from a ewe shortly after abortion possesses a high titre against *V. foetus* there is a strong probability that the abortion was associated with this organism. On the other hand, the absence of agglutinins in significant amount provides little evidence that the abortion was not vibrionic. This is manifest (Table I) in the results obtained with the sera from certain ewes which aborted but failed to exhibit a significant rise in antibody; furthermore, of three sera from ewes which aborted in the flock from which strain VC was isolated, two only produced a trace of agglutination with VC at a titre of 1 : 20 and none agglutinated VS significantly. From some of the instances quoted, it is evident that the reason for this absence of agglutinins is obscure, though in other cases the low titres would appear to be due to the strain used for the test being different in some respect from that which caused the abortion (*vide* Tables III and IV). Plastridge and Williams (1943) have noted this absence of agglutinins in cows which have aborted from infection with *V. foetus*. The low titre has also been attributed by some workers to using a newly isolated strain for preparing antigen. Although no direct confirmation of this latter point was obtained, it may be pointed out that all strains were subcultured several times before the antigen was prepared. Taking into account both the possible difference of antigenic structure among strains of *Vibrio foetus* and the fact that the serum of ewes which have aborted recently does not necessarily agglutinate even the strains isolated from the outbreak, the agglutination test cannot be recommended for the routine diagnosis of vibrionic abortion though there might be occasions in which it would be of some ancillary value.

II. SOME OBSERVATIONS ON THE ANTIGENIC RELATIONSHIP BETWEEN STRAINS OF *V. foetus*

In the previous section the results of agglutination tests on sera obtained from flocks in which vibrionic abortion had occurred suggested that antigenic differences exist between strains of *V. foetus*. In order to obtain further information on this point, it was decided to study further the antigenic relationship of four strains—VS, VT, VB and VC. It would have been desirable to have investigated a greater number but this was not possible owing to the difficulty of isolating and maintaining cultures of this organism and to the small number isolated even in laboratories handling a large volume of sheep material.

Methods.—(1) *Preparation of H sera.* Agglutinating sera were prepared against two strains by immunising rabbits with merthiolated antigen of an opacity equal to Brown's tube No. 8. The method adopted was to give four daily injections of one drop, suspended in 1 c.c. of saline, repeating the procedure with double the preceding dose at intervals of three days.

(2) *Preparation of O sera.* The O-antigens for use in the production of agglutinating sera were prepared by suspending the organisms in absolute alcohol and heating for 30 minutes at 56° C. and, after removal of the alcohol, proceeding as in the case of H-antigens.

(3) *Suspensions for agglutination tests.* Suspensions of *Vibrio foetus* antigen killed by merthiolate, as previously described, were employed. The principal results obtained were confirmed using suspensions to which no preservative had been added in order to exclude the possibility that it might have affected the reactions.

The relationship between somatic antigens. Agglutination tests were carried out with sera prepared against the O-antigens of strains VB, VS and VC. The titres to which these sera agglutinated the three strains and strain VT are shown in Table V, from which it is evident that the strains are not homologous although there is some relationship between VB and VT. Unfortunately it was not possible to prepare an O-serum against VT owing to the loss of this strain.

TABLE V

THE TITRES OF ALL STRAINS AGAINST SERA PREPARED WITH ALCOHOL-TREATED ANTIGENS

Serum prepared against:	*Antigen*			
	VB	VS	VC	VT
VB	320	0	0	80
VS	0	320	0	0
VC	0	0	320	0

The relationship between flagellar antigens. Sera prepared against the motile strains VS and VT were tested against all four antigens. These results are shown in Table VI, from which it is evident that there is considerable cross agglutination between the two motile strains VT and VS, whereas non-motile VC is agglutinated by neither serum and non-motile VB is agglutinated by VT serum at a relatively low dilution of 1 : 80. It has been shown that the O-antigens of VB and VT are related so that some cross agglutination was to be anticipated. Though the H-antigens of VS and VT were agglutinated to the same titre by serum VS, it could not be concluded that they were identical because they were agglutinated by serum VT to different titres.

TABLE VI

THE TITRES OF ALL STRAINS AGAINST SERA PREPARED WITH THE MOTILE STRAINS VS AND VT

Serum prepared against:	*Antigen*			
	Motile		*Non-motile*	
	VS	VT	VB	VC
VS	5,120	5,120	0	0
VT	320	1,280	80	0

The differences between sera prepared against motile strains before and after treatment with alcohol lead inescapably to the conclusion that alcohol destroys some part of the antigenic structure of the organism. Unfortunately, in carrying out agglutination tests with *V. foetus* it was found that the rate of agglutination and kind of floccules formed did not afford a means of distinguishing between the types of agglutination but, by analogy with the Salmonella group, it has been assumed that treatment with alcohol destroys the flagellar antigen. To justify this assumption, it is necessary to show that the antigenic loss induced by treatment is consequent also upon loss of motility without treatment. Unfortunately, no antigen corresponding to VB, when it was motile, was available when this question came under consideration. To determine this point the motile strain VS was subcultured several times on blood-agar slopes to render it non-motile and the growth washed off to prepare antigen (A1). At the same time inoculum from the slopes was seeded into peptone water and incubated, the resultant growth which was motile being centrifuged to prepare antigen (A2). These antigens were then tested against the serum prepared against the motile strain VT, the H antigens of which had been found to be closely related to VS. It was found that A1 was not agglutinated but that A2 was, the titre being expressible as 1 : 20 to 1 : 80 +++, 1 : 160 ++. This is a reasonably high titre indicating the presence of antigens corresponding to agglutinins present in serum prepared against VT ; that the agglutination was not complete may have been due to some of the organisms not having recovered their motility. It was found that alcohol treatment had no effect on the antigens of the non-motile strains and the above results are therefore consistent with the view that alcohol destroys the flagellar antigen though it cannot be regarded as a proved conclusion.

Strains of *V. foetus*, when grown on solid medium, usually produce two colonial forms, one being comparatively large (2-3 mm.) and the other minute. Subcultures from either produce a growth in which the same colonial form tends to be more abundant, though it was impossible to obtain a pure growth of either. Antigens were prepared with each type of colony from strain VS and it was found that neither of these antigens was agglutinated by serum prepared against VT though each when seeded on peptone water produced a motile growth which was agglutinated by this serum. It was therefore concluded that neither of these colonial variants was connected with the loss of H-antigen in growths on solid medium.

DISCUSSION

It is obviously not possible to form an adequate picture of the antigenic structure of *V. foetus* from an examination of only four strains ; nevertheless it may be concluded that all strains are not homogeneous and that there are type-specific O-antigens. Further-

more, while there was evidence of antigenic relationship between the flagellar antigens of the two motile strains examined, the O-antigens of two of the four strains, as judged by tests on alcohol treated suspensions, were completely heterologous. This observation confirms the view that routine diagnostic tests carried out against a single laboratory strain are of very little value.

One of the confusing features of the results was the fact that we were unable to show any relationship between strains VS and VB from two different outbreaks, although the sera from the affected sheep showed some cross agglutination. This would suggest some underlying antigenic factor common to the strains although the possibility of infection of sheep with a multiplicity of antigenic types cannot be disregarded.

SUMMARY

An attempt was made to diagnose vibrionic abortion by the agglutination test using sera from infected sheep and an antigen composed of a motile strain of *V. foetus*. It was found that, whereas samples from normal sheep gave a titre of 1 : 20 or less, most of those from infected animals agglutinated the organism to a titre of more than 1 : 40.

Observations over a period revealed that the titre was highest immediately after the act of abortion.

In some infected sheep, however, the titre was less than 1 : 40. In view of this finding and the narrow limit between the maximum normal titre and the average titre in the majority of infected sheep the agglutination test cannot be recommended for the routine diagnosis of vibrionic abortion.

By using alcohol treated antigens it was possible to establish that differences exist in the somatic antigens of different strains. The bearing of this observation on the practical application of the agglutination test is discussed.

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STUDIES IN LOUPING-ILL
III.—LOUPING-ILL ANTISERUM

by

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INTRODUCTION

In recent years it has been widely recognised that in the virus infections of man and animals, immunity response by the host tissues corresponds well with general immunological theories and results, as in the bacterial infections, in the appearance of specific antibodies in the blood stream. The importance of this is considerable for, in the control of many virus affections, specific antisera prove of great prophylactic value and in some instances are used extensively for both active (serum-virus) and passive immunisation.

Since neutralising antibody, capable of titration (Schwentker, Rivers and Finkelstein, 1933; Burnet, 1936, etc.), appears in the serum of animals that have received parenteral injections of louping-ill virus, it was thought important to explore the possibility of preparing antisera with a view to their use for the prophylactic control of the seasonal outbreak of the disease in lambs. Further, with knowledge derived from an investigation of the properties of the antibody neutralising louping-ill virus, it was hoped to attempt later an investigation into active immunisation in which, by a method or methods of antibody titration, the antigenic values of different vaccines could be determined.

With these objects in mind experiments were undertaken on the production and titration of louping-ill antisera, and some of these, as representatives of the whole, are described in this paper.

PRODUCTION AND TITRATION OF LOUPING-ILL ANTISERA

Sera for examination were obtained from sheep which had survived experimental louping-ill and from sheep and horses which had received large and repeated doses of active virus. In the case of sheep, homologous brain-virus and culture-virus administered by subcutaneous and intramuscular injection were used as antigens in separate experiments. Culture-virus was available in large quantities but otherwise had no advantage over brain-virus. The latter, even in large doses, was well tolerated; abscesses did not develop but, occasionally, slight fibrous thickening of the subcutaneous connective tissues developed at sites of multiple injections. Horses received either mouse brain-virus or culture-virus administered subcutaneously. Mouse rather than sheep brain-virus was used to prevent the formation in horses of antibodies to sheep brain-tissue proteins as it was not known whether the presence of such antibodies in sera would be entirely non-injurious to lamb recipients.

In one experiment, two horses which received active virus as a first injection each developed a mild but distinct febrile phase five days later and virus was demonstrated in the blood of one of them. On the ninth day after the injection, the other—a thoroughbred stallion—exhibited nervous symptoms indicative of encephalomyelitis. The animal died after observable illness of about 12 hours. In the surviving horse it was soon evident that mouse brain-tissue virus was unsatisfactory for hyperimmunisation since each injection was followed, usually within a period of 48 hours, by an abscess-like swelling at the site of injection. These swellings were not considered to be due to pyogenic infection and may have represented the individual's reaction to the tissue cells and proteins of the mouse brain. In other experiments with horses, however, culture-virus was satisfactory and quantities up to 400 c.c. (10^6 M.I.D. of virus per c.c.) were administered with no apparent ill-effect.

It was usual to inject virus on successive days but occasionally the doses, generally three in number, were given over a period of four or five days. After a rest period of 10 to 14 days the course of injections was repeated.

No rapid test suitable for estimating the degree of immunity that existed in an animal at a particular time was available, and following the work of others, bleedings were made usually on the seventh and tenth day after the last dose of virus. Individual bleedings from the jugular vein consisted of 400 c.c. to 500 c.c. in the case of sheep and approximately 7 litres in horses.

In attempts to estimate the amount of immune body present in the sera of hyperimmunised animals, three methods were employed, *viz.* :

In vitro neutralisation, in which serum and virus were mixed and allowed to interact for a period of three hours before being administered by intracerebral injection into mice.

Titration of the inhibitory action of immune serum on growth of virus in chick embryo tissue plus Tyrode's fluid culture-medium.

In vivo neutralisation, in which serum and virus were simultaneously injected on opposite sides of the body.

Individually, or in relation to one another, the tests formed a measure or standard by which antibody values have been assessed to sera before issue for test against natural infection in the field.

In vitro NEUTRALISATION

This method was used by Schwentker *et al.* (1933) as a test for the presence of immune bodies in the serum of monkeys which had received several intraperitoneal injections of active louping-ill virus. In the present work the test has proved of great value but slight modifications of the original technique have been introduced.

Virus obtained by cultivation in chick embryo tissue, plus Tyrode's fluid, was found to be more suitable than mouse or sheep

brain-virus. This was probably due to the fact that the suspension was water clear, or nearly so, and yet as rich in virus content as suspensions of brain material. The use of normal saline solution for preparing the virus dilutions was discontinued; instead, normal sheep serum obtained from sheep whose sera were known to possess no antibody was used as diluent to form, as in the original technique, a scale of tenfold dilutions from 1 in 5 to 1 in 50,000. Equal quantities of the serum under test and virus or appropriate virus dilutions were mixed as in the original method. No alterations in the subsequent technique were made except that 0·05 c.c. of the mixtures of virus and serum instead of 0·03 c.c. were injected intracerebrally into the test mice. With each group of unknown sera, ranges of virus dilutions against two or more normal sera were customarily tested.

Standardisation of louping-ill immune sera.—Reference to Table I shows that sera of high antibody value may be obtained in sheep by hyperimmunisation with sheep brain-virus and in sheep and horses by the use of cultivated virus. Thus, in the titration of serum No. 1 mice receiving a 10^{-1} virus dilution survived an intracerebral injection of 10,000 infective doses of virus (the amount determined by corresponding titrations of normal sera Nos. 2 and 3). Serum No. 1, therefore, in 0·025 c.c. amounts contained sufficient antibody to neutralise 10,000 infective doses of virus.

This calculation is based on the assumption that 0·05 c.c. of the highest dilution of virus (in the presence of normal serum) which produces louping-ill in at least 50 per cent. of the mice represents one mouse infective dose (1 M.I.D.). Approximate antibody values of sera obtained from immune and hyperimmune animals were then calculated, the standard antibody unit being fixed arbitrarily as that amount of serum which neutralised 100 mouse infective doses of virus.

As indicated above, 0·025 c.c. of hyperimmune serum No. 1 (Table I) neutralised 10,000 M.I.D. of virus, and thus this quantity contained 100 units of neutralising-antibody or, to adopt the expression customarily used when evaluating bacterial antitoxins, 4,000 units of antibody per c.c.

Samples of serum from sheep and horses hyperimmunised with culture-virus contained as much, and on some occasions apparently more, antibody per c.c.

Titration of the Inhibitory Action of Immune Serum on Growth of Virus in Tissue Culture-Medium

Preliminary experiments showed that immune serum added to the medium (chick-embryo tissue plus Tyrode's fluid) not only inhibited the growth of the virus added as inoculum but also destroyed it. It is not known whether virus destruction took place

rapidly or slowly during the period of incubation of the cultures. It was evident, however, that the effect was due to the contained antibody and not to inherent properties of sera as such, since in control experiments good growth of virus was readily obtained in cultures containing from 10 per cent. to 50 per cent. concentrations of normal serum.

The technique adopted was to add decreasing quantities of immune serum to a series of flasks containing freshly prepared medium. A constant quantity of virus inoculum was then added to each flask, and after suitable incubation at 37° C. for three to five days, the presence or absence of virus in each flask was determined by the intracerebral injection of 0·1 c.c. of the undiluted culture into mice.

When two series of cultures, in which the corresponding flasks contained the same graded quantities of immune serum, were incubated after receiving one and two volumes of virus inoculum respectively, it was found that in the first series the amount of immune serum necessary to inhibit growth and destroy virus was only half that required in the second series (Table II).

One fact concerning the properties of louping-ill immune sera has emerged. It will be noted in Table II, under column *Series* 1, that the three cultures which contained 0·25 c.c. of hyperimmune serum were devoid of virus after incubation ; *i.e.*, 0·25 c.c. of serum neutralised 2 c.c. of virus inoculum, a quantity much greater than that neutralised when mixtures of immune serum and virus were injected intracerebrally into mice. A mixture of 0·025 c.c. hyperimmune serum and 0·025 c.c. virus in the form of undiluted culture nearly always produced louping-ill on intracerebral injection of mice whereas one containing 0·025 c.c. serum and 0·005 c.c. virus was generally neutral or nearly so (see Table I). Apparently eight, or possibly 40, times more virus was neutralised in tissue culture-medium than in mixtures of immune serum and virus inoculated intracerebrally into mice.

Comparative titrations.—In view of the fact that the virus neutralising property of an immune serum as determined by the one method failed to show even an approximate quantitative relationship to the neutralising property as determined by the other, further parallel experiments embracing both titration methods were carried out. Each culture, containing hyperimmune sheep serum or a graded quantity of its dilution in normal sheep serum, received 2 c.c. of virus inoculum and was incubated for three or four days at 37° C. Immediately after the cultures had been prepared, mixtures of the same immune sera and the identical virus inoculum were made for *in vitro* neutralisation tests. The results of three such experiments are given in Table III.

Reference to the upper part of Table III (Method 1 : Titration) shows that, quantitatively, the virus-neutralising property of antisera was proportional to the amount of contained antibody. When, as

in the case of No. C, immune serum was diluted 1 in 10 or 1 in 100, proportionally less virus was neutralised. Undiluted serum No. C (0·025 c.c.) neutralised 10,000 M.I.D. of virus, and it is possible that this is not the maximum since intermediate titrations with virus concentrations of between 1 in 2 and 1 in 10 were not made. When, however, the immune serum was diluted 1 in 10, the amount of virus neutralised was not more than 1,000 M.I.D., and at dilution of 1 in 100 possibly not much more than 100 M.I.D.

In neutralisation in " tissue culture " (Method 2, Table III), where immune sera or their dilutions were titrated against a fixed quantity of virus, the amount of serum required had to be increased in proportion to the dilution. Thus 0·5 c.c. of serum A undiluted, or 5 c.c. of 1 in 10 dilution, was more than was necessary for complete neutralisation of 2 c.c. virus inoculum, but with half the quantities (0·25 c.c. and 2·5 c.c.) of the respective antisera neutralisation was incomplete. The results obtained with sera B and C were very similar. Normal sera in control cultures possessed no power to prevent survival or growth of virus.

The experiments recorded in Table III confirmed the belief already expressed that more virus was neutralised by immune serum in tissue culture-medium than in the simple *in vitro* mixtures tested by injection into the mouse brain. If No. C serum is taken as an example, it will be seen that in Method 1, 0·025 c.c. undiluted serum neutralised 10,000 M.I.D. of virus : 1 c.c. of serum, therefore, would neutralise 400,000 M.I.D. of virus. By Method 2, 0·5 c.c. of the same undiluted serum (No. C) neutralised 2 c.c. inoculum which contained 4,000,000 M.I.D. of virus. One c.c. of the serum, therefore, would neutralise 8,000,000 M.I.D., *i.e.*, 20 times more virus than the same volume of serum would neutralise by injection of serum-virus mixtures into the mouse brain.

The difference is remarkable and cannot be explained on the assumption that there is much immediate loss or deterioration (19/20) of the virus inoculum in a " tissue culture " as it has been shown (Wilson, 1945) that in tissue culture-medium particularly small quantities of virus are capable of survival and growth. It is possible that the phenomenon represents a rapid formation of antigen-antibody molecules or a greater stability of these in an environment of the various types of cells present in tissue culture-medium in contrast to that of purely nervous tissue cells which, according to theory, limit themselves to a type of metabolism that does not include any function the mechanism of which is concerned with immunity or its development. A closely similar phenomenon has been noted by other workers where virus-neutralising capacity of a particular antiserum may apparently vary according to the indicator tissue used in the test. Thus, Andrewes (1928, 1930), Fairbrother (1932) and Goyal (1935) working with vaccinia virus record that a serum-virus mixture which is neutral when tested by intradermal injection can show activity on intracerebral or intratesticular injection.

In vivo NEUTRALISATION

This series of experiments has comprised solely a study of the neutralisation of virus in the tissues of the sheep. Serum was given on one side of the body and virus on the other each by subcutaneous injection. As a rule the doses were administered simultaneously, but occasionally virus was given 24 hours after serum. The amount of virus in the suspension (sheep brain-virus) used as a test dose was estimated by intracerebral injections in mice. Under the controlled conditions, different antisera were evaluated in terms of their ability to neutralise *in vivo* the standard (100 M.I.D.) or multiples of the standard dose of virus.

In sheep, louping-ill virus after subcutaneous injection first attacks cells of the visceral system, and in most cases evidence of infection is represented by the development of fever concurrently with the appearance of virus in the blood stream. Recovered animals are immune even to virus introduced into the brain. In titrations, therefore, the values of antisera are based on their ability to prevent multiplication of virus as evidenced by the absence of thermal response and failure of virus to appear in the blood.

In a small proportion of experiments the interpretation of the result has been difficult; in some, this may have been due to the wide fluctuations in body temperature which occur normally in sheep, while in others intercurrent infections were probably responsible. In cases in which suspiciously high temperatures developed blood samples were taken for subinoculation tests in mice. As a rule this procedure sufficed to indicate whether the febrile response resulted from virus which had escaped the action of immune serum. When doubt still remained, final confirmatory evidence as to the source of the febrile response could be obtained since it was found that when a serum was sufficiently potent to neutralise *in vivo* a test dose of virus administered subcutaneously, the brain still remained susceptible, whereas control sheep, *i.e.*, those which had received virus only, were immune to virus introduced into the brain.

The results obtained in the examination of individual and pooled samples of sera are presented below.

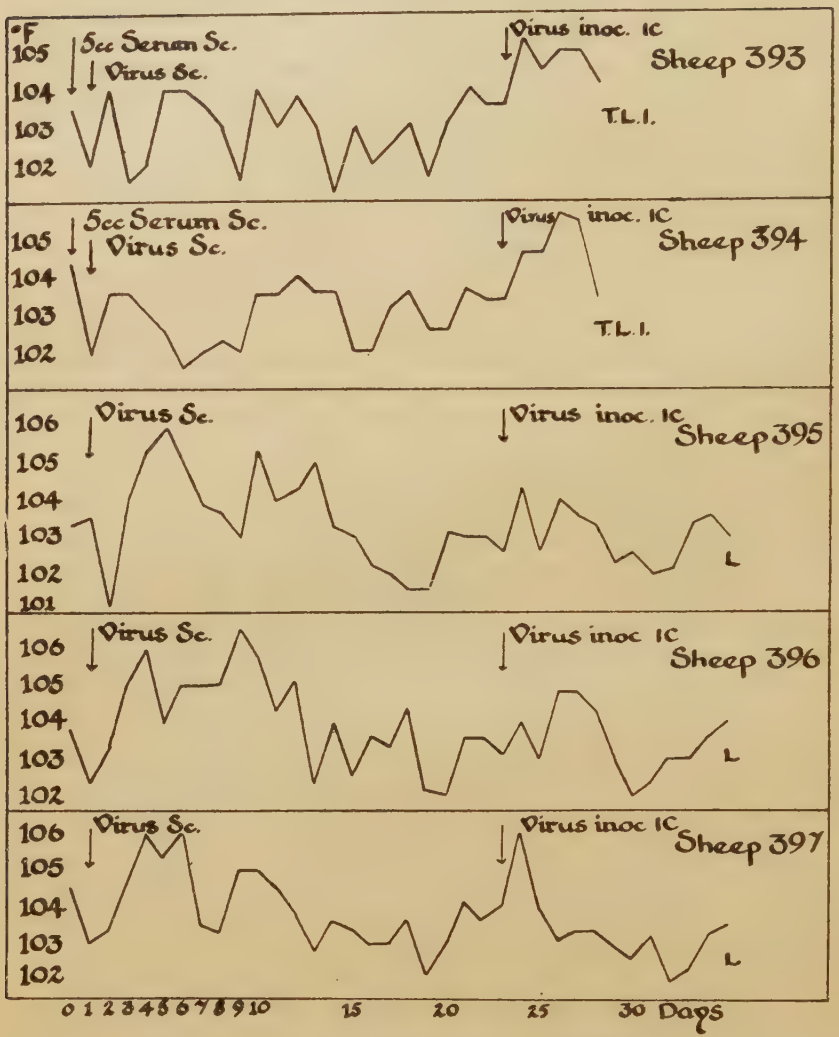
Sera from hyperimmunised sheep

Homologous brain-virus

Experiment 1.—Five c.c. of immune serum obtained from an individual sheep after ten hyperimmunising injections of virus, was injected subcutaneously into each of two normal sheep (Nos. 393 and 394). On the following day both, together with three controls (Nos. 395, 396 and 397), were given 20,000 M.I.D. of sheep brain-virus. The injections were made subcutaneously on the opposite side of the body and the temperature recorded daily. (Chart 1.) While the temperature of sheep 394 remained fairly steady during the period of observation, that of 393 was irregular, fluctuating

CHART I

In vivo neutralisation of louping-ill virus with antiserum



Abbreviations : Sc. = Administered subcutaneously.
IC. = Administered intracerebrally.
TLI. = Typical louping-ill.
L. = lived.

between 101° F. and 104° F. All the controls became infected with temperature readings of 106° F. or over.

Twenty-two days after the injections all five animals were given approximately 4,000 M.I.D. of virus by intracerebral inoculation. As was expected, Nos. 395, 396 and 397 were immune but both serum-treated animals developed louping-ill, 393 dying in six days

and 394 in five. The existence of louping-ill in No. 394 was confirmed by histological examination of cerebrum, cerebellum and medulla, and by the demonstration of virus in high titre in these tissues. No. 393 was not examined.

It therefore appeared that 5 c.c. of immune serum given subcutaneously was sufficient to prevent the development of an active infection in sheep following the injection of 20,000 M.I.D. of virus but consequent on the failure of virus to become active, the tissues of the central nervous system remained susceptible to louping-ill.

Since interpretation of the test may be difficult on temperature readings alone, the demonstration of susceptibility of the brain under the circumstances mentioned has proved of considerable value and has subsequently been applied not only in the work on determination of potency of antisera but also with respect to that method of testing the antigenic value of vaccines where a precise interpretation of a temperature reaction following administration of the test dose of virus is important.

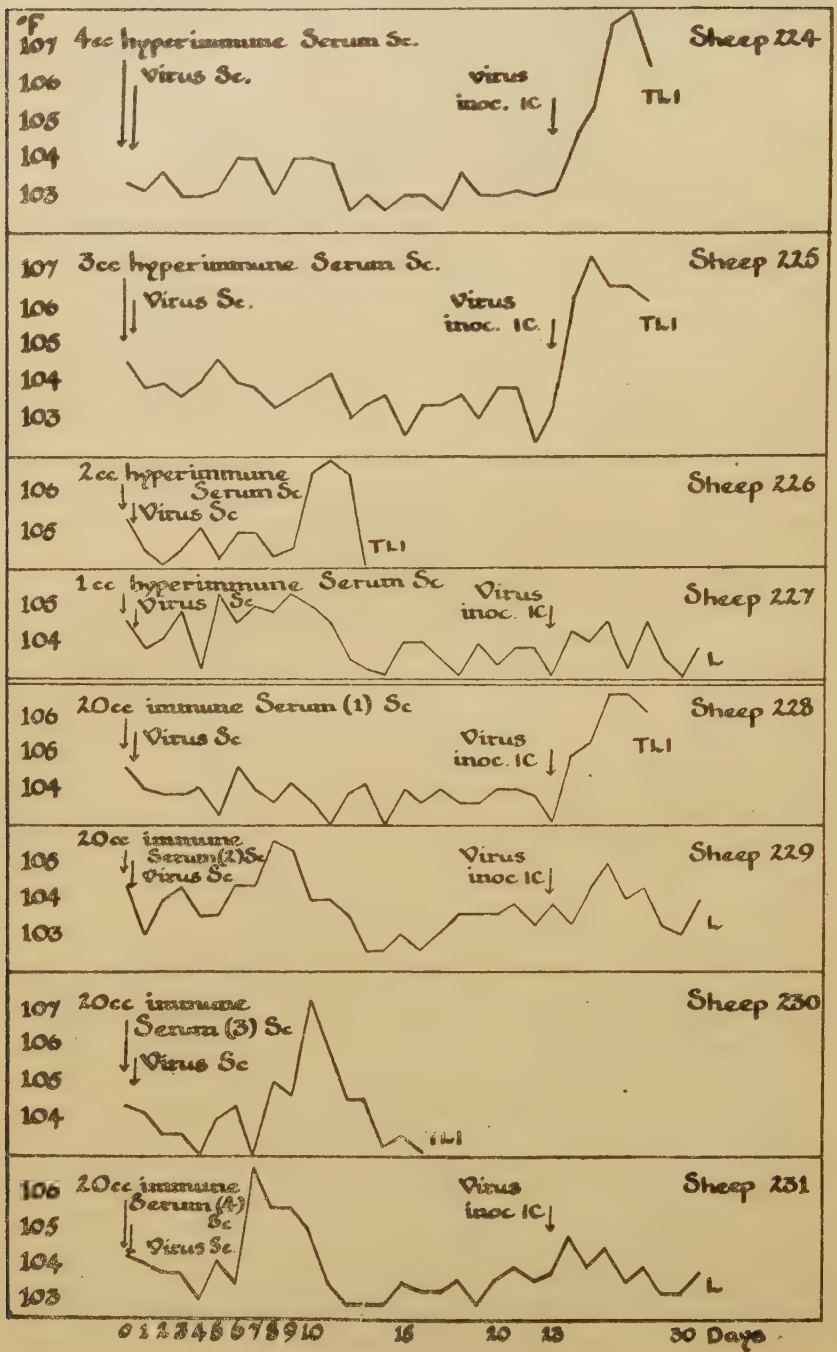
Experiment 2.—This experiment was concerned with the more accurate titration of an antiserum of known antibody value as determined by an *in vitro* neutralisation test (Method 1, where mice received serum-virus mixtures intracerebrally).

Hyperimmune serum of 4,000 antibody units per c.c. was administered subcutaneously in doses of 4 c.c., 3 c.c., 2 c.c. and 1 c.c. respectively to sheep Nos. 224, 225, 226 and 227. In addition, four samples of immune serum obtained from sheep which had recently survived experimental infection, containing approximately 400 units of antibody per c.c. (sample No. 1) and 40 units antibody per c.c. (samples 2, 3 and 4) respectively, were included in the same test. They were injected in 20 c.c. doses to sheep Nos. 228, 229, 230 and 231 respectively. The dose of virus, given simultaneously, was large and probably exceeded 100,000 M.I.D. Daily temperature recordings were taken and on the 23rd day, after the administration of serum and virus, the immunity of the central nervous system was tested by the intracerebral injection of virus.

Reference to Chart 2 in which the temperature records of the individual sheep are recorded, shows that Nos. 224 and 225 were respectively protected by 4 c.c. and 3 c.c. of hyperimmune serum. Sheep No. 226, which had received 2 c.c. of serum, was protected against virus in some degree in that there was no early febrile response and therefore apparently no immediate multiplication of virus in the visceral tissues. The high temperatures recorded on the tenth, eleventh and twelfth days were possibly due to activity of virus which had escaped the action of antiserum and had gained entrance to the central nervous system. Concurrently with the rise in temperature, nervous symptoms were exhibited and the animal died from typical louping-ill. The remaining animal (No. 227), which had received 1 c.c. hyperimmune serum, developed only a mild temperature reaction: it showed symptoms of brain involvement

CHART 2

In vivo titration of hyperimmune sheep serum



Abbreviations: As in Chart 1.

on the ninth and tenth days but recovered. Of the four sheep (Nos. 228, 229, 230 and 231) that received immune serum, only one (No. 228) showed no thermal response and of the remaining three, one (No. 230) died from louping-ill.

Although the experiments indicated that the interpretation of the test from temperature records alone was possible, the surviving animals were injected intracerebrally with approximately 20,000 M.I.D. of virus. The results confirmed the view already formed, namely, that 4 c.c. and 3 c.c. hyperimmune serum and 20 c.c. immune serum (sample No. 1), protected the visceral tissues against the activity of virus; in consequence, in Nos. 224, 225 and 228, the brain and spinal cord tissues had remained susceptible and death occurred after the intracerebral injection of virus. On the other hand, animals 227, 229 and 231, which showed a febrile response to virus administered subcutaneously, were immune to subsequent intracerebral injection.

If 2 c.c. of hyperimmune serum (4,000 units antibody per c.c.) is accepted as being the approximate dose which will protect a sheep against 100,000 M.I.D. of virus, it is not surprising that 20 c.c. of sample No. 1, containing 400 units of antibody per c.c. was similarly protective, nor could the failure on the part of samples Nos. 2, 3 and 4, which contained only 40 units of antibody per c.c., be regarded as unusual since, in each case, a calculated dose of 200 c.c. serum would have been required.

As in the tests with mice where previous interaction between antibody and virus was allowed *in vitro*, the amount of virus which could be neutralised by antisera *in vivo* in sheep appeared to be directly proportional to the antibody content. This does not mean that a particular antiserum would neutralise the same amount of virus by both methods of titration. *In vivo*, titration experiments showed that approximately 2 c.c. of hyperimmune serum neutralised 100,000 M.I.D. of virus, which in terms of units of antibody per c.c. represents 500 units. This quantity is only one-eighth of the amount of antibody found when mixtures of the same serum and culture-virus were made *in vitro* and subsequently injected into the brains of mice. It should, however, be pointed out that the figure 100,000 M.I.D. was calculated on the basis of 0·05 c.c. of a 10^{-5} dilution representing 1 M.I.D. whereas in fact two out of six mice receiving the next higher virus dilution (10^{-6}) developed typical louping-ill. It is possible that the test sheep, instead of receiving 100,000 M.I.D., received an amount of virus approximating 1,000,000 M.I.D.

Culture-virus

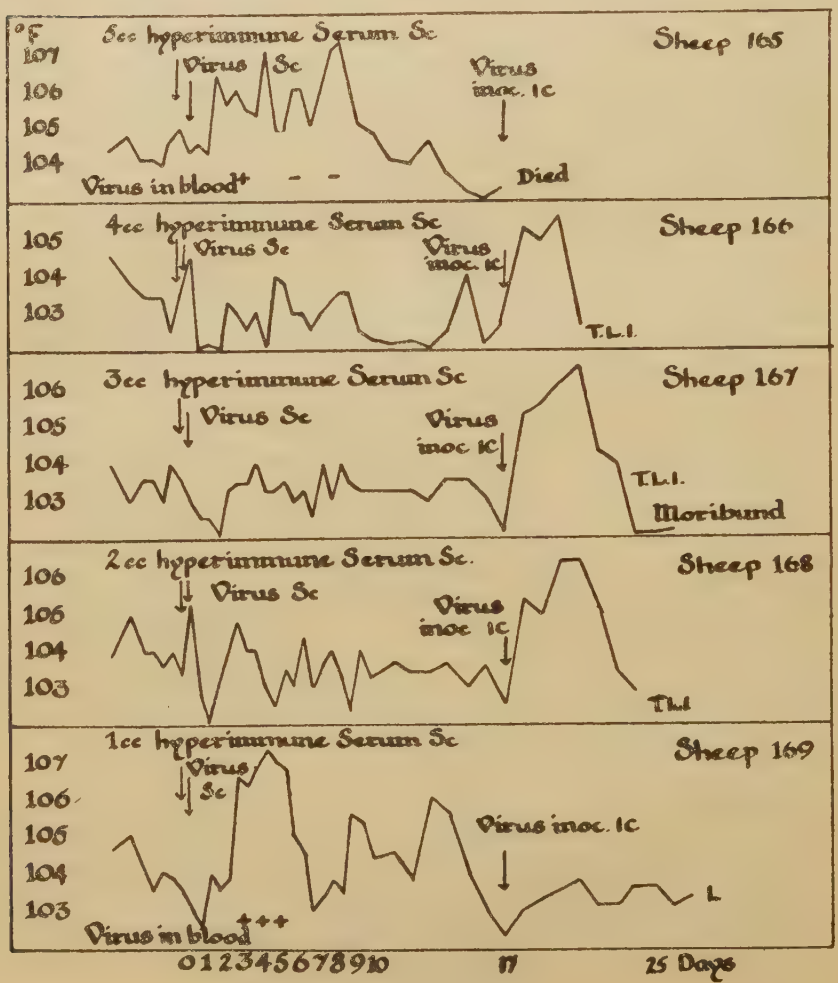
Experiment 3.—The single *in vivo* neutralisation experiment performed with serum obtained from sheep hyperimmunised with culture-virus was carried out in a manner similar to that of Experiment 2. The serum consisted of a representative pool from each sheep and from each bleeding made during the course of hyper-

immunisation. While the experiment primarily concerned titration of the smallest quantity of serum that would protect against a standard quantity of virus, active immunisation was being studied at the time and the opportunity was taken to determine the antigenic response, if any, in sheep receiving serum plus virus. On this account the same group of experimental sheep are referred to in a subsequent paper dealing with active immunisation.

A dose of brain-virus approximating 1,000,000 M.I.D. was administered six hours after the serum. Thereafter, temperature recordings were made twice daily for nine days and then once daily until the 17th day, when 20,000 M.I.D. of virus was injected into the brain. The records of the individual sheep appear in Chart 3.

CHART 3

In vivo titration of louping-ill hyperimmune sheep serum



Abbreviations: As in Chart 1.

With one exception, sera of 4,000 antibody units per c.c. from sheep hyperimmunised with culture-virus, protected in a manner comparable to that of sera from sheep hyperimmunised with brain virus. The exception occurred in the case of Blackface ram No. 165, which remained unprotected although it had received the largest dose (5 c.c.) of serum. Three of the remaining sheep (Nos. 166, 167 and 168), which respectively received 4 c.c., 3 c.c. and 2 c.c. immune serum, were apparently protected, while sheep No. 169, which developed a typical febrile phase, was unprotected with 1 c.c. serum. Virus was present in the blood stream on three consecutive days and when tested on the 17th day by intracerebral injection of virus, No. 169 sheep was completely immune. In contrast, Nos. 166, 167 and 168 were susceptible. In one (No. 167) symptoms of louping-ill did not appear until the seventh day after intracerebral injection of virus, and it is noteworthy that in this animal the infection appeared to affect principally the lower part of the spinal cord, for posterior paralysis was the main symptom.

Blackface ram (No. 165), on the evidence previously outlined, should have been immune to intracerebral virus, but on the day following injection it died. Brain trauma is a possible explanation of its death although no convincing evidence of this was found on *post-mortem* examination.

With the exception of No. 167, the type of louping-ill which resulted from the intracerebral injection of virus into sheep which had previously received immune serum plus virus in appropriate amounts, was indistinguishable clinically from that resulting from the primary injection of virus into the brains of normal sheep. In all such cases which were examined, lesions were widely distributed throughout the central nervous system; moreover, in most cases virus titres in the same tissues were uniformly high. A feature of distinction, however, was that in the former type virus failed to appear in the blood stream during the period of fever and on no occasion was it found in the spleen after death. The presence of virus in the blood and spleen following intracerebral injection into normal sheep has previously been recorded (Pool *et al.*, 1930; Greig *et al.*, 1931; Gordon *et al.*, 1932). This difference might be explained on the assumption that, although serum completely inactivates virus, either some degree of visceral passive immunity remained or in these tissues active immunity, resulting from the administration of serum plus virus, was present at the time virus was injected into the brain.

Serum from hyperimmunised horses

Mouse brain-virus

Two of the three experiments performed are summarised in Table IV.

In the first experiment, sheep Nos. 52, 56, 57, 59 and 60, which received 5 c.c., 5 c.c., 10 c.c., 40 c.c. and 80 c.c. of immune serum

respectively, were found to be protected against the test dose (100,000 M.I.D.) of virus. In the second experiment, Nos. 62, 64, 65 and 66 were protected by 10 c.c. immune serum against the same test dose of virus. As with homologous immune serum, protection was considered to have been established when virus failed to stimulate reaction on the part of the host tissues ; fever did not develop nor did virus gain access to the tissues of the central nervous system. In subsequent intracerebral tests for immunity, susceptibility of the brain and spinal cord tissues was found to exist and at death virus was usually found to be abundant in the same tissues, although on rare occasions it may be absent, as in the case of sheep No. 57, which showed pronounced louping-ill symptoms on the fifth and sixth days followed by coma for several days before death ensued. The apparent failure of 20 c.c. of serum to protect sheep No. 58, when 10 c.c. and 5 c.c. of the same serum had completely protected Nos. 57 and 56 respectively, and also the failure of 10 c.c. serum to protect sheep No. 63 after its ability to do so in the case of No. 62, indicates some irregularity in the action of the heterologous serum in protecting sheep. The term " failed to protect " is perhaps only partly true as the general impression formed was that, in cases where temperature reactions and even death occurred, considerable protection was afforded by the immune serum. This impression was based on the fact that three out of four serum-treated sheep died following a thermal response which was delayed in onset and which, from the examinations made, appeared to be due to virus attack on the brain. Thus, almost concurrently with the rise in temperature in these cases (Nos. 58, 63 and 67), there occurred symptoms of brain dysfunction referable to virus attack on that organ. After death virus was found in brain or in pooled brain and cord tissue, and lesions considered to be typical of louping-ill were found in all.

The inference was drawn that in sheep there existed a tendency for virus to escape destruction by heterologous antiserum and that, in such circumstances, the purely nervous type of louping-ill frequently developed.

Culture-virus

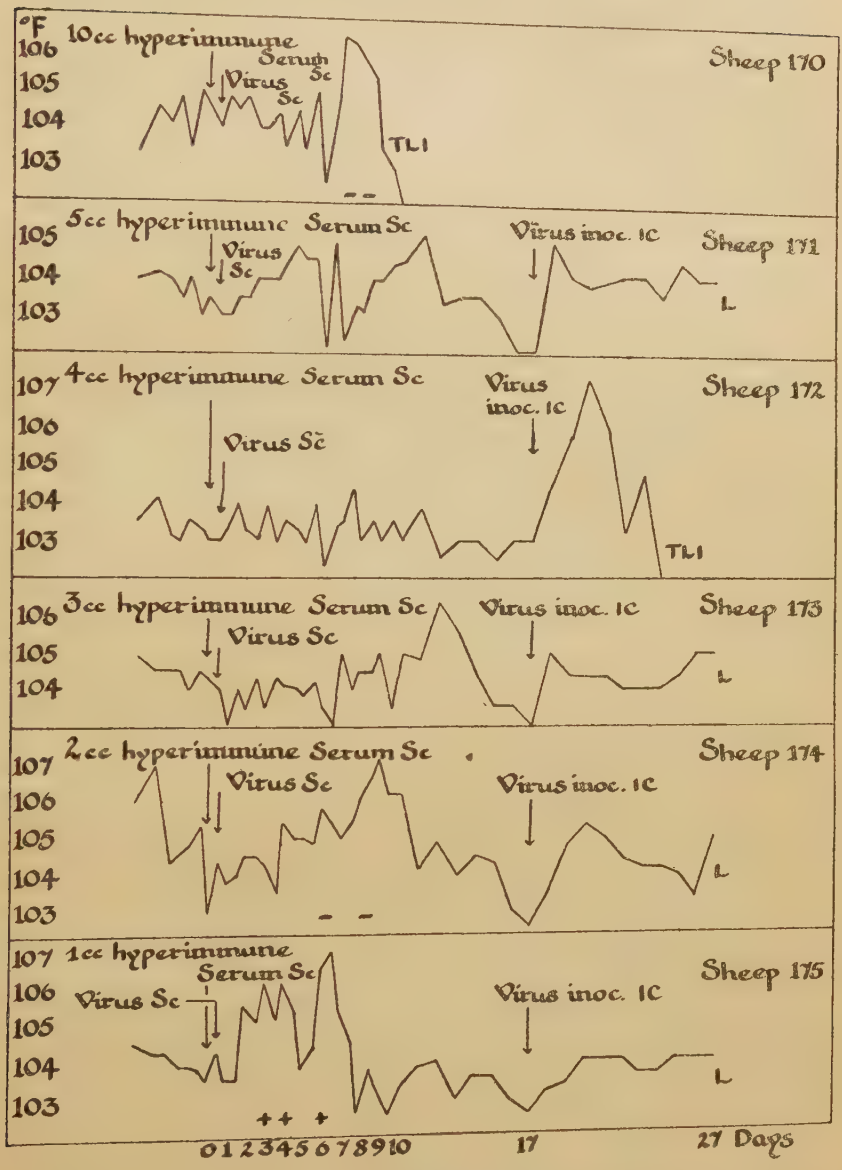
In like manner as the trials with the corresponding type of immune serum obtained from sheep, one experiment was made with the dual purpose of titration and determining the antigenic response in recipients of immune serum plus virus.

Pooled serum containing approximately 4,000 antibody units per c.c. (Method 1, Titration) was injected subcutaneously in graded doses into sheep Nos. 170, 171, 172, 173, 174 and 175. Approximately 1,000,000 M.I.D. of virus was administered six hours later and thereafter temperature records kept. (Chart 4.)

With the reservation that again heterologous immune serum was probably irregular in its action, the results, when viewed generally, indicated that serum containing 4,000 antibody units per c.c. pro-

CHART 4

In vivo titration of louping-ill hyperimmune horse serum



Abbreviations :
+ = Virus present in blood stream.
— = Virus not detected.
Others as in Chart 1.

tected in a manner fairly comparable to that of homologous serum of the same titre. The irregularity of heterologous serum is noted

by the protection of No. 172 (Chart 4) by 4 c.c., whereas 5 c.c. and 10 c.c. of the same serum only gave partial protection to Nos. 171 and 170 and virus entered the brain tissues of at least one of them (No. 170). In this animal the reaction elicited was typical of the delayed type mentioned in the previous experiment and the rise in temperature appeared to be due wholly to the action of virus in the brain and spinal cord. On the other hand, in sheep No. 175 which received 1 c.c. of hyperimmune serum, the reaction was due to activity of virus in the visceral tissues and was typical of that produced in sheep receiving virus alone by the subcutaneous route. In this instance the rise in temperature occurred early and virus was present in the blood on three occasions. The evidence obtained from No. 174 indicates that 2 c.c. of serum also failed to protect, for a febrile period commenced possibly as early as the fourth day and, although two tests failed to detect virus in the blood stream, multiplication in the visceral tissues probably occurred.

It became evident in some instances that it was difficult to interpret results in terms of ability or inability of virus to multiply in the visceral tissues. For example, in No. 173 the immunity demonstrated by intracerebral injection of virus may indicate that multiplication previously occurred in the visceral tissues or in the brain or in both, during the period of fever which commenced about the seventh day and reached its peak on the twelfth. As will be recorded in later studies, the development of high serum-antibody titres in sheep receiving serum plus virus was found to be associated with virus activity in visceral sites as opposed to low or very low antibody titres in cases in which no evidence of virus multiplication could be obtained or where the evidence indicated that activity of virus occurred solely in the central nervous tissues. Thus, the antibody content of serum obtained from No. 173 on the day intracerebral injection was performed (17 days after subcutaneous administration of serum plus virus) was low—no higher than that of the corresponding sample obtained from No. 172. In contrast to this, high antibody titres equivalent to those in survivors of a subcutaneous dose of virus, were found in the corresponding sera of Nos. 174 and 175.

Although, during the seventh to fourteenth day period, no brain symptoms were noted in Nos. 173 and 171, the brain immunity which was demonstrated was probably due to virus, having gained entrance to, and multiplied solely in, the brain tissues during the period of primary infection.

That louping-ill infections of the central nervous system may occur in sheep without the appearance of concomitant louping-ill symptoms has not been subjected to experimental proof, but in other neurotropic virus diseases this phenomenon occurs and Hurst (1936), by subinoculation experiments, demonstrated the presence of equine encephalomyelitis virus in the cerebro-spinal fluid of one experimental monkey which, at the time of test, appeared normal, and

TABLE I

TITRATION OF LOUPING-ILL ANTIBODY IN SERA OF SHEEP AND HORSES

Virus dilution in normal sheep serum		*Serum under test*		*Final virus dilution*	*Serum under test*						
					1	2	3	4	5	6	7
0·5 c.c. culture	+	0·5 c.c. serum	=	1 in 2	D	5	5	7	6		
					8	5	5	7	8		
					9	6	5	9	10		
					10	6	6	14	S		
0·5 c.c. 1 : 5 culture	+	0·5 c.c. serum		10^{-1}	D	6	6	S	9		
					S	6	6	S	S		
					S	6	6	S	S		
					S	7	6	S	S		
0·5 c.c. 1 : 50 culture	+	0·5 c.c. serum	=	10^{-2}	S	6	6	S	S		
					S	6	6	S	S		
					S	7	7	S	S		
					S	7	7	S	S		
0·5 c.c. 1 : 500 culture	+	0·5 c.c. serum	=	10^{-3}	D	D	7			5	6
					S	6	7			6	6
					S	7	7			6	6
					S	7	8			6	7
0·5 c.c. 1 : 5,000 culture	+	0·5 c.c. serum	=	10^{-4}	S	7	7			6	6
					S	8	7			6	6
					S	8	7			7	7
					S	9	8			7	7
										7	7
										10	8
0·5 c.c. 1 : 50,000 culture	+	0·5 c.c. serum	=	10^{-5}	S	8	7			D	8
					S	8	8			6	8
					S	11	11			8	9
					S	S	S			8	S
										9	S
										S	S

Serum 1 = Pooled serum from sheep after hyperimmunisation with brain-virus.
Sera 2 and 3 = Normal sera acting as controls in test on serum 1.
Sera 4 and 5 = Sheep and horse serum respectively after hyperimmunisation with culture-virus.
Sera 6 and 7 = Normal sheep and normal horse serum respectively acting as controls in tests on Sera 4 and 5.
Figure = Destruction of the mouse when showing typical symptoms of louping-ill × days after inoculation.
D = Death from undetermined cause.
S = Survival for period of 15 days.

TABLE II

TITRATION OF TWO SAMPLES OF SERUM FROM SHEEP HYPERIMMUNISED WITH BRAIN-VIRUS

	Culture Series 1					*Culture Series* 2			
	Amount immune serum present					*Amount immune serum present*			
	1 c.c.	0·5 c.c.	0·25 c.c.	0·125 c.c.	*None*	1 c.c.	0·5 c.c.	0·25 c.c.	0·125 c.c.
Immune Serum A	S, S, S, S, S, D, S, S, S	S, S, S, S, S, S, S, S, S	S, S, S, S, S, D, S, S, S	8, 10, 12, 12, S, 7, 8, 8, 8	6, 6, 7, 7, 7	S, S, S, S, S, S, S, S, S	S, S, S, S, S, S, S, S, S	10, 10, S, S, S 7, 8, 8, S	D, 8, 8, 9, 10, 6, 7, 8, 9
Immune Serum B	S, S, S, S	S, S, S, S	S, S, S, S	11, S, S, S	5, 5, 6, 6	S, S, S, S	S, S, S, S	D, S, S, S	9, 10, 12, S

Cultures of 50 c.c. volume. Those in Series 1 received 2 c.c. and those in Series 2 received 4 c.c. virus inoculum. Presence or absence of Virus in the individual cultures determined by mouse intracerebral test.
Abbreviations as in Table I.

TABLE III

COMPARATIVE TITRATION OF HYPERIMMUNE SERA AND THEIR DILUTIONS

(1) *In vitro* Neutralisation, Mice then receiving 0·05 c.c. intracerebrally

Virus dilution	*Serum under test*									
	Serum A and control			*Serum B and control*			*Serum C and controls*			
	Serum A	*Serum A.* 1 *in* 10 *dilution*	*Normal serum*	*Serum B*	*Serum B.* 1 *in* 10 *dilution*	*Normal serum*	*Serum C*	*Serum C.* 1 *in* 10 *dilution*	*Serum C.* 1 *in* 100 *dilution*	*Normal serum*	*Normal serum*
1 in 2	7	6		7	7		7	8			
	10	7		7	7		7	8			
	S	7		8	7		10	8			
	S	7		8	8		13	9			

10^{-1}	D S S S	6 7 11 12		8 9 S S	7 7 8 11		S S S S	7 8 10 13	6 7 7 8		
10^{-2}	8 S S S	S S S S		S S S S	7 S S S		S S S S	9 S S S	D 9 9 12		
10^{-3}	S S S S	S S S S	6 6 7 7 7	S S S S	S S S S	5 5 6 6	S S S S	S S S S	S S S S	D 6 6 7	7 7 8
10^{-4}			7 7 8 9 10			6 6 6 6			S S S S	7 7 7 7	D 7 8 8
10^{-5}			8 14 S S S			6 6 7 7				8 9 S S	7 8 14 S
10^{-6}										S S S S	D S S S

Sera A, B and C obtained from sheep hyperimmunised with culture-virus.

(2) Neutralisation of virus in "Tissue Culture"

	Cultures receiving undiluted hyperimmune serum				*Cultures receiving 1 in 10 dilution of hyperimmune serum*							*Cultures receiving 1 in 100 dilution of hyperimmune serum*			*Control cultures receiving normal serum or no serum*			
	1 *c.c.'s*	0·5 *c.c.'s*	0·25 *c.c.'s*	0·125 *c.c.'s*	10 *c.c.'s*	5 *c.c.'s*	2·5 *c.c.'s*	1·25 *c.c.'s*	1 *c.c.'s*	0·5 *c.c.'s*	0·25 *c.c.'s*	0·125 *c.c.'s*	50 *c.c.'s*	25 *c.c.'s*	12·5 *c.c.'s*	50 *c.c.'s*	25 *c.c.'s*	*None*	*None*
Serum A and controls	S, S, S, S	S, S, S, S	7, 7, 8, 11	6, 6, 6, 7	S, S, S, S	S, S, S, S	S, 7, 8, 8	7, 7, 8, 9	6, 7, 7, 7,	D, 6, 6, 7	4, 5, 5, 5	4, 4, 5, 5	—	—	—	—	—	4, 4, 5, 5	4, 5, 5, 5
Serum B and controls	S, S, S, S	S, S, S, S	D, 9, S, S	6, 7, 7, 7	S, S, S, S	S, S, S, S	S, 8, 9, 9	5, 5, 6, 6	5, 5, 5, 6	4, 4, 5, 5	D, 4, 4, 5	D, 4, 5, 5	—	—	—	—	—	4, 4, 5, 5	4, 4, 4, 5
Serum C and controls	S, S, S, S	S, S, S, S	7, 8, 9, 10	5, 6, 7, 7	S, S, S, S	D, S, S, S	6, 8, 8, 8	5, 5, 6, 6	—	—	—	—	S, S, S, S	S, S, S, S	6, 8, 8, 8	5, 5, 6, 6	4, 5, 5, 5	4, 4, 5, 5	4, 5, 5, 5

The fluid portion of the cultures was made up to 50 c.c. with Tyrode's fluid or to 100 c.c. in the case of cultures receiving 50 c.c. of serum. Cultures incubated for 3 to 4 days then tested in mice which received 0·1 c.c. undiluted culture.
Abbreviations as in Table I.

TABLE IV

In vivo NEUTRALISATION OF VIRUS WITH SERUM FROM HORSE HYPERIMMUNISED WITH MOUSE BRAIN-VIRUS

Exp. No.	*Sheep No.*	*Dose immune serum Sc.*	*Dose of virus Sc.*	*Result*			*Interval between virus injections*	*Intracerebral test for developed immunity approx. 20,000 M.I.D. of virus being given*				
				Temperature reaction	*Presence of virus in the blood*	*Nervous symptoms*		*Temperature reaction*	*Presence of virus in the blood*	*Symptoms*	*Post-mortem examination*	
											Presence of virus in the brain	*Presence of virus in the spleen*
1	52 } 5	c.c. serum obtained after 21st virus injection	100,000 M.I.D.	—		None	21	+	—	T.L.I.5	+	
	53		,,	+	—	+ 11th day with death on 13th day†						

	54, 55	5 c.c. serum obtained after 24th virus injection	„	—		None	21	+	—	None		
	56	5 c.c.*	„	+ (delayed)	—	None	21	—		None		
	57	10 c.c.*	„	—		None	21	+	—	T.L.I.5	+	
	58	20 c.c.*	„	+ (delayed)	—	None	21	+	—	T.L.I.5	—	
	59	40 c.c. *	„	—		+ 9th day with death on 10th day†						
	60	80 c.c. *	„	—		None	21	+	—	T.L.I.5	+	
	61		„	+		None	21	+	—	T.L.I.5	+	
						None	21	—		None		
2	62, 63	10 c.c. serum obtained after 21st virus injection	100,000 M.I.D.	—		None	23	—		T.L.I.5	+	—
			„	+ (delayed)	—	+ 8th day with death on 9th day†						
	64, 65	10 c.c. serum obtained after 24th virus injection	„	—		None	23	+		T.L.I.5	+	—
	66	10 c.c. serum obtained after 28th virus injection	„	—		None	23	+		T.L.I.5	+	—
	67		„	+ (delayed)	—	None	23	+		T.L.I.5		—
						+ 9th day with death on 12th day†						
	69		„	+	+		23	—		None		
	70		„	+	+		23	—		None		

* Pooled serum obtained after 13th, 18th, 21st and 24th virus injections.

Abbreviations : Sc. = Subcutaneous.
M.I.D. = Mouse infecting doses.
† = Virus present in the brain tissues.
T.L.I.×. = Typical louping-ill × days after inoculation.

subsequently remained so. Recently Sabin and Ward (1941) recorded the occurrence of 16 cases of non-paralytic experimental poliomyelitis in monkeys, diagnoses having been made by histological examinations of the nervous tissues in all and by the demonstration of the virus in some. They point out that non-paralytic poliomyelitis infection is almost invariably associated with the destruction of an appreciable number of nerve cells in the spinal cord, but that the infection, symptomless as it is, fails to progress on account of the setting up of an equilibrium between the host and the virus.

DISCUSSION

In reviewing the work concerning the titration of antibodies, the evidence indicates that any one of three methods may be adopted as a means of assessing the quantities of immune body present in sera from louping-ill immune and hyperimmune sheep, *viz.* (1) mouse intracerebral test; (2) estimation of the inhibitory action of immune serum on the growth of virus in tissue culture-medium; and (3) *in vivo* neutralisation of virus in sheep. Although the antigen in each test has of necessity been the active, disease-producing virus, titrations have been sufficiently accurate to reveal a direct proportional relationship between the amount of antibody present in sera and the amount of virus neutralised thereby.

In calculating the antibody value of an immune serum on the basis of the amount of virus neutralised, there were differences in the three methods; as little as 1/20 of the amount of virus was apparently neutralised by the first method as by the second, while, compared with the third, the first showed a closer relationship in regard to this property. Such differences in the apparent value of an immune serum as were found were probably related to the tissue environment to which antibody and virus were subjected, since, by examining considerable numbers of sera, it was noted that the ratios of values as determined by one method were the same as the ratios of the values as determined by the other two.

The first method of titration is readily applicable to the examination of large numbers of sera; the second, which is less suitable in this respect, might under certain circumstances be of use in the examination of sera of very low antibody content, as, for example, the sera of animals receiving a single dose of inactive virus vaccine; the third, although having limitations under laboratory conditions, is obviously important and essential in the examination of sera in relation to the possible passive immunisation of lambs. If the time factor concerning the duration of passive immunity is taken into consideration, and it is also assumed that the pathogenicity of virus transmitted by infective broods of ticks (MacLeod and Gordon, 1932) is not significantly different from virus transmitted experimentally in a mechanical manner from one animal to another, it is reasonable to expect that results similar to those obtained experi-

mentally by the third method might be obtained in field tests. If this is the case, then the results obtained with sheep sera might be viewed favourably since it is unlikely that any one animal in the field would receive from ticks an amount of virus equal to that used experimentally for the titration of sera.

Only in the respect that large supplies of antigen were readily available had culture-virus any advantage over homologous brain-virus in hyperimmunisation of sheep; in the horse, however, the use of culture-virus was an improvement since it eliminated troublesome local reactions which arose when mouse-brain virus was used.

In the first two methods of titration, sheep and horse immune sera were similar in action, but the results obtained with horse sera tested *in vivo* in sheep were disappointing. If the fact that the sera were titrated against quantities of virus (10^5 and 10^6 mouse infecting doses) probably much greater than that transmitted by ticks, is excluded, it seems reasonable to assume that horse immune sera used prophylactically in the field will give irregular results; indeed, it is possible they will be even more irregular than was found by experiment, since there could be no guarantee of infection occurring at the time passive immunity was greatest.

From the farmers' point of view it is immaterial whether lambs receiving heterologous immune serum develop louping-ill in a form in which the virus attacks the brain and cord alone. From the epizootiological aspect, heterologous serum may have considerable prophylactic value, for by the passive immunisation of visceral tissues the frank cases occurring in the field might represent a form of the disease in which virus was sequestered in the central nervous system. In the absence of multiplication of virus in the visceral tissues or escape of virus not immediately neutralised in the visceral system from the brain and cord, engorging ticks would have a diminished opportunity of ingesting blood containing the virus necessary for subsequent transmission of the infection.

SUMMARY

Louping-ill immune sera may be obtained from sheep and horses hyperimmunised either with brain-virus or with virus cultivated in medium consisting of chick embryo tissue plus Tyrode's fluid.

Immune sera may be titrated by any one of three methods, (*a*) the injection of serum-virus mixtures into the brains of mice; (*b*) estimation of the inhibitory action of immune serum on growth of virus in tissue culture-medium, or (*c*) by *in vivo* neutralisation of virus in sheep, which indicates that louping-ill virus is neutralised to a degree proportional to the amount of antibody contained in the sera.

The virus-neutralising capacity of an immune serum, as determined by injection of serum-virus mixtures into the brains of mice, has been expressed in terms of units of antibody per c.c. of the

serum, one unit having been chosen as representing the amount of antibody capable of neutralising 100 mouse infective doses of virus.

In the different methods of titration, a lack of fixed relationship between antibody and antigen, suggested as being due to the tissue environment to which these were subjected, has been observed with regard to the virus-neutralising capacity of a serum. By method (*a*) approximately 1/20 of the amount of virus was neutralised as by method (*b*); while method (*a*), when compared with method (*c*), showed a much closer relationship regarding the virus-neutralising capacity of an antiserum.

The ratios of the antibody values of immune and hyperimmune sera as determined by method (*a*) were the same as the ratios of the values as determined by methods (*b*) and (*c*).

The subcutaneous injection to sheep with antiserum in appropriate amount given simultaneously with virus suppresses the activity of virus in the visceral tissues, but by so doing prevents the development of immunity in the central nervous tissues.

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EXPERIMENTAL INFECTIONS IN VIRGIN HEIFERS WITH *TRICHOMONAS FOETUS* IN VACCINATED AND UNVACCINATED ANIMALS

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INTRODUCTION

It was demonstrated in earlier work (Kerr and Robertson, 1941) that a high titre of antibody against *Tr. foetus* could be induced in cows by intramuscular vaccination. The experiments described in this paper were undertaken to ascertain if the circulating antibody produced in virgin heifers by intramuscular vaccination afforded protection against the subsequent infection of the animals with *Tr. foetus* at insemination. The methods employed in assessing infection included the serum agglutination test (Robertson, 1941), the intradermal skin test with extract of Trichomonas (Kerr, 1944) and direct examination for the presence of the protozoan in the genital tract.

HISTORY OF ANIMALS

The animals used fall into three groups according to the treatment they received before infection. Groups I and II (designated with the letter D) were bought when six months old and grazed on pasture completely isolated from other cattle and from other grazing grounds. They were observed for one year before being infected, and vaccination of those selected for the purpose was carried out in the last months of this period. The animals were all about 18 months old when exposed to infection.

Group I.—D.2 and D.11 were controls and received no treatment before infection.

Group II.—D.4, D.6, D.7 and D.8 were vaccinated three times by intramuscular injection, commencing when they were 13 to 14 months old. They received 1,000 millions of living *Tr. foetus* and 2,000 millions at ten-day interval, followed after three and a half months by 4,000 millions.

Group III.—A.17, B.17, A.18, A.11c, 1943; A.9c, 1943, were virgin heifers which, as newly born calves, had received the colostrum of mothers which had high titre sera from previous vaccination. This had resulted in a temporary blood titre against *Tr. foetus* in the calf and the skin also became sensitive to the intradermal injection of antigen. In all cases, however, blood and skin reactions were negative for at least six months before infection was undertaken. Thus this group comprises a further set of controls.

The individual histories of these animals are as follows.

*A.*17 was born on April 20th, 1942, and was the daughter of a repeatedly vaccinated heifer which was exposed to infection by placing living *Tr. foetus*

in the vagina before service by a clean bull. The heifer did not become infected and the calf which was born at normal term was healthy. It acquired a high passive blood titre from the colostrum, but this had disappeared at ten months. The skin test had become negative eight months before exposure to infection. This heifer was two and a half years old when exposed to infection.

*B.*17 was born on May 5th, 1943, and was out of a normal cow from a clean herd. The maternal colostrum was withheld and the calf given whey from the colostrum of a vaccinated animal together with boiled milk. The whey had only a moderate titre (15 : 9) and B.17 showed a low positive titre against *Tr. foetus.* There was a positive skin reaction, but both this and the blood titre were transitory and had disappeared by the time the calf was four months old. This heifer was 18 months old when exposed to infection.*

*A.*18, which was born on March 11th, 1943, was also the calf of a normal mother from a clean herd. The cow was vaccinated intramuscularly with a single dose of Trichomonas 17 days before calving but the response was slight. The calf was fed on the colostrum and developed a low blood titre of short duration. The skin test became negative between the fourth and fifth month.

This heifer was one year eight and a half months when exposed to infection.

*A.*11*c*, 1943, was born on June 21st, 1943. The mother had been exposed to infection at service in 1941 (the service of the previous pregnancy), but infection did not occur and she calved normally. During 1942–1943 the cow was vaccinated intramuscularly with a large number of doses of a Trichomonas extract and also had sterile Trichomonas extract instilled into the uterus after calving in 1942. She was served by an infected bull on September 24th, 1942, but showed no sign of infection and a healthy calf, A.11c, 1943, was born. The calf was given the maternal colostrum and developed a high blood titre. At six months, however, the titre of the serum was not above the normal level. The skin test had become negative in eight months. The mother of A.11c, 1943, was thus twice exposed to infection but did not acquire the disease.

This heifer was one year five and a half months old when exposed to infection.

*A.*9*c*, 1943, which was born on March 14th, 1943, was the calf of normal mother never exposed to infection. The mother had been vaccinated and had a high serum titre. The calf was fed on the maternal colostrum and in consequence acquired a high passive titre in its serum, but this had reverted to the normal at least ten months before exposure to infection while the skin test had been negative for at least eight months.

This heifer was one year nine months old when exposed to infection.

The freedom of virgin heifers from *Tr. foetus* infection has been placed in some doubt by Andrews and Miller (1936), who reported finding the parasite in such heifers in an infected herd. This is the only observation of its kind which has so far been made and they

* It should be recalled that the blood agglutination titre is expressed by giving the total number of crosses awarded in the first four dilutions ($\frac{1}{6}$ $\frac{1}{12}$ $\frac{1}{24}$ $\frac{1}{48}$) over the number awarded in the same dilutions of a normal serum with the same suspension on the same day. The reasons for this convention are given in detail in previous papers (Robertson, 1941; Kerr and Robertson, 1941, 1943, 1946.)

themselves felt at the time that infection might have been conveyed to the heifers through the medium of contaminated instruments.

During the early study of this disease, one of the present writers (W. R. K.) made a practice of seeking Trichomonas by direct examination of virgin heifers running with infected animals on infected farms but obtained no evidence of its presence. He also endeavoured to infect a number of virgin and non-pregnant animals (a total of 20) by the direct instillation of material from infected animals into the vagina but was never successful in setting up infection. The view taken by the present writers is that infection resulting from physical contamination under natural conditions in virgin animals must be extremely rare and is probably very transitory. They are not fully convinced of the natural occurrence of the infections described by Andrews and Miller (1936).

In the present experiments the animals in Groups I and II were never in contact with infected animals at any time before infection was attempted at the time of insemination. Those in Group III were kept in separate quarters removed from all other stock until they were about four months old. They were then allowed to mix with calves of similar ages until they were between six and eight months old. After this they were turned out to grass with the general herd of the station in which there were no acutely infected animals. The animals of this group were under regular observation and were repeatedly blood tested and skin tested at intervals. Direct examination of the vagina was not undertaken. That there are no tests for latent infection in virgin animals will be seen when the nature of the disease is followed in the sequel.

Trichomonas does not invade the blood stream and, in the experience of the present writers, congenital infection does not occur in live calves. They also consider that the healthy calf of a mother exposed to infection which does not take, is in the same case as the normal calf of a non-exposed mother.

From the nature of the disease, which, in the writers' view, is due to a uterine and not a vaginal parasite, it is concluded that virgin heifers do not carry a latent infection.

After insemination and exposure to infection all three groups of heifers ran together.

MATERIALS AND METHODS

Insemination was performed with 2 c.c. of semen undiluted except for the addition of living Trichomonas, introduced with a glass pipette into the uterine cervical canal. The inseminations were made as nearly as could be judged at the middle of the heat period. The animals were under constant supervision and none was inseminated when not in oestrum. In all, except D.2 of group I and D.4 of group II, a washed suspension containing from 5 to 10 millions of *Tr. foetus* was mixed with the otherwise undiluted semen

before injection into the cervical canal. The semen and the Trichomonas were used as quickly as possible after obtaining them. In the cases of D.2 and D.4, insemination was made with undiluted semen and exposure to infection was carried out four hours later by introducing 100 million Trichomonas in 5 c.c. of saline solution into the cervix.

RESULTS

In the following account the word " take " is used to indicate the setting up of an infection whereby Trichomonas could be found by direct examination on one or more occasions at any period after the fourth or fifth day from the introduction of the protozoa: in other words there was evidence of multiplication and establishment as distinct from a brief survival of the flagellates originally introduced. The word " pyometra " is used to designate those cases in which there was an effusion into the uterus of an exudate rich in cells most of which were of the epithelial type. The term is not defended as being altogether suitable. The exudate was rich in Trichomonas and the condition was always an indication of acute infection. Its amount varied in different cases but the description pyometra has only been applied when the uterus became much enlarged and tense and the condition culminated in the emission of a milky discharge followed by expulsion of the foetus. Culture, by all the customary methods, of both uterine fluid and material taken from various sites in the foetus failed to reveal *C. pyogenes* or other bacteria (cultural examination was carried out in two of the three pyometra cases). *Tr. foetus* was readily cultivated from all abortion cases. In a previous group of five pyometra cases uncomplicated by other infection, which were slaughtered for the purpose of studying the histology of the endometrium, the only change found was a gross oedema with an increase of histiocytes. In the pyometra cases of the present experimental group of animals a similar uterine reaction was observed.

Nature of the Infection and the Type of Disease Produced

Group I. Controls (D.2 and D.11). D.2 was inseminated and Trichomonas was introduced four hours later. The heifer did not become infected and had a normal pregnancy. D.11 was infected at the time of insemination but the resulting disease was of a low-grade type. The heifer returned to oestrum at 13 days and was re-inseminated but no Trichomonas was included in the semen on this occasion. Conception failed and the animal became anoestrus. The low-grade type of infection which it displayed is of some practical importance and will be referred to later.

Group II. Vaccinated animals (D.4, D.6, D.7 and D.8). D.4 was exposed to infection by introducing the Trichomonas four hours after insemination. As parasites were never seen after the first day,

it was considered that infection had not taken place. The heifer returned to service at three weeks and was re-inseminated but no further Trichomonas was introduced. Conception occurred, followed by a normal pregnancy. D.6, D.7 and D.8 all became infected when the protozoa were introduced at insemination. They aborted at 16, 14½ and 18 weeks respectively. D.6 and D.7 developed pyometra before abortion and the uterine discharge which was milky in appearance, non-mucoid and cellular, showed *Tr. foetus* in great numbers. The uterus rapidly returned to normal after abortion had taken place (24 to 36 hours), the vaginal secretion being clear, mucoid and non-cellular. Although D.8 did not develop pyometra before abortion there was a profuse discharge with large numbers of Trichomonas afterwards. The uterus of this animal continued to be hard and tense for three days, after which it returned to normal. In each case the foetus was obtained. In D.6, which aborted at 16 weeks, it was 6 inches long, and in D.7, aborting at 14½ weeks, 2 inches. Cultures from the foetal fluid, membranes, abomasum and heart blood gave no bacterial growth, while *Tr. foetus* was isolated without difficulty. D.8, which aborted at 18 weeks, expelled a foetus 2 inches long. Cultures were not made in this case.

Group III. These animals were not vaccinated but, as already described, had a temporary passive agglutination titre in early calfhood which had disappeared at least six months before exposure to infection. They were all exposed to infection at insemination. A.17 and B.17 became infected and developed the disease in an acute form. A.17 aborted at about eleven weeks but the foetus was not found. Pyometra developed before abortion and there was a milky fluid discharge containing a large number of Trichomonas. The uterus at this time had become much enlarged and tense and the foetus could not be palpated in it. Special reference must be made to B.17 for it was infected with strain " Manley," which differs serologically in some respects from strain " Belfast " (Kerr and Robertson, 1945) which was used in the other cases. There was no obvious pyometra before abortion occurred at the 13th week. Cultures, on blood plates and in broth, from the placental fluid, the membranes and the abdominal contents of the foetus, showed no bacterial growth. Trichomonas was isolated from the foetus by cultivation. A.18 and A.11c, 1943, both developed infection but in a low-grade form; pregnancies were not interrupted and they both calved at full term. Infection was clearly established in these two heifers but showed the subclinical form produced in the " C " experiments of earlier work (Kerr and Robertson, 1943). In parenthesis, it should be stated that the Trichomonas used for A.18 was a passage strain raised from the vagina of the acutely infected D.8 (group II) which was infected with Belfast strain. The last heifer of group III (A.9c, 1943) did not become infected; Trichomonas was never found and the animal calved normally.

Thus of a total of eleven heifers exposed to infection, three, one from each group, failed to take. Of the eight infected, five aborted between the 11th and 18th week while the remaining three had definitely established infections though of a low-grade type. One of these three (D.11) failed to conceive on re-insemination and then became anoestrus and the other two (A.18 and A.11c, 1943), although developing the disease in a subclinical form, carried their calves normally.

The main results are given in concise form in Table I and the details of each animal are set out in Table II.

TABLE I

Previous treatment	*No. of animal*	*Method of infection*	*Result*	*No. of successful infections*	*Total exposed*	*Remarks*
Group I	D.2	After insem.	Does not take: calves at full term.	1	2	Note method of attempted infection in D.2.
None.	D.11	*At* insem.	Takes: does not conceive.			
Group II. Vaccinated.	D.4	*After* insem.	Does not take: calves at full term.			Note method of attempted infection in D.4.
Antibody circulating at time of infection.	D.6 D.7 D.8	*At* insem.	Aborted.	3	4	Vaccination in degree used afforded no protection against infection at insemination.
Group III.			A.17 and B.17 aborted.			
Temporary passive calfhood titre.	A.17 B.17 A.18	All *at* insem.	A.18 infection takes; but calves at full term.	4	5	Two of the successfully infected, A.18 and A.11c, have uninterrupted pregnancies.
	A11. c		A.11c, inf. takes but calves at full term.			
	A.9c		A.9c, inf. does not take; calves full term.			
			Totals	8	11	8 infected. 5 abort. 1 does not conceive. 2 have successful pregnancies.

Presence of Trichomonas in Vaginal Secretion

There was no clear correlation between the frequency with which Trichomonas was observed and the form of the disease. In general, parasites increased in number shortly before abortion but there was considerable variation. In one respect, however, the findings in all the acutely infected animals were in agreement, namely, the disappearance of the Trichomonas after abortion. In none of the five animals which aborted between the 11th and 18th weeks of pregnancy was Trichomonas found later than 48 hours afterwards. This is the general but not invariable finding, for in one case from a field outbreak at Banbridge, Co. Down, the parasites were found for some months after abortion.

Hammond and Bartlett (1945) have recently noted the persistence of Trichomonas in heifers infected for the first time by service from an infected bull. All their animals returned to heat after an average period of 25 days. They state that " the last positive sample appeared between 72 and 126 days after coitus, that is through two to four oestrus cycles subsequent to the first return to oestrum."

Among the low-grade infections set up in the present experiments, it should be noted that in D.11 (group I) Trichomonas was present at the return of oestrum at 13 days. The parasites persisted in the uterus and were seen again at six weeks, when the animal had become anoestrus. Trichomonas in small numbers have been found in the uterus but not in the vagina of a proportion of non-pregnant animals examined at an abattoir. Their histories were unknown but the findings suggest that the parasites sometimes show a tendency to persist in the uterus.

The Skin Reaction and the Agglutination Titre of the Blood

Before the findings in the infected animals are considered reference should be made to one or two interesting points which were revealed during the course of vaccination. It will be recalled that vaccination consisted of two intramuscular injections of living Trichomonas (1,000 millions and 2,000 millions) given at an interval of ten days. A third dose (4,000 millions) was given three and a half months later. Infection was carried out about one month after the final dose, and it was expected that this would be the time of highest " immunity." All four animals showed a positive blood titre after two doses, but they did not react in exactly the same way. D.6 and D.8 had consistently high titres, whereas D.4 and D.7 did not respond so well. The skin tests behaved in much the same way; D.6 and D.8 showed marked reactions, while D.4 and D.7 were less sensitive. The effect of the third vaccination was interesting; it raised the blood titre in all cases but its influence on skin sensitivity was different. About the time of the third injection D.4 had a skin test reading of 10-19, but on receiving the third dose the skin became desensitised with a reading of 9-10·5, which is below the

TABLE II

Heifers	*Vaccinated or not*	*Other previous treatment*	*How infected*	*Trichomonas seen*	*Titre of blood serum*	*Skin reaction*	*Result*	*Remarks*
Group I								
D.2	*Not*	None	*After* insemination 100×10^6 in saline into uterus.	Only seen up to 4th day.	Not above normal.	Not positive.	Infection does not "take." Animal calves at full term.	
D.11	*Not*	None	At insemination.	Seen for first 17 days and again for one day at 6 weeks.	14½:9 low positive at 9 weeks, previously negative.	Low positive for short time.	Returned at 13 days. Did not conceive when re-inseminated; infection "takes."	No trichomonas introduced at second insemination. Animal becomes anoestrus.
Group II								
D.4	Vaccinated 3 doses	None.	*After* insemination	Only seen on first day.	Positive titre from vaccintion but not very high.	Positive skin test from vaccination.	Returned at 3 weeks. Re-inseminated without reinfection; in calf. Infection failing to "take."	Normal calf at full term.
D.6	Vaccinated 3 doses.	None	At insemination.	Not seen until 6 weeks, after which seen frequently; very numerin pyometra fluid during *last* 14 days. Disappeared after abortion.	Positive titre from vaccination, high titre produced.	Very marked positive reaction from vaccination but becomes less marked for 14 days before abortion. Sensitive skin response reappears after abortion.	Aborts at 16 weeks. Foetus 6 inches. *Pyometra* develops before abortion.	Note reduction in skin reaction at development of pyometra.

D.7	Vaccinated 3 doses	None.	At insemination but re-insem. and re-infect. after 3 weeks.	Not seen during first attempt at infection. At second insem. and re-infect.; frequently seen after 7th day until abortion. Not seen after abortion.	Varying titre from vaccination.	Tendency to have variation in skin reaction from vaccination, also during infection, particularly when pyometra develops.	Aborts at 14½ weeks. Foetus of about 8 weeks old. Pyometra before abortion.	Immune response showed tendency for titre to drop during vaccination.
D.8	Vaccinated 3 doses	None.	At insemination.	Seen frequently for the first month but not again until day of abortion. Disappeared after first day after abortion.	Consistently high titre from vaccination.	Consistently strong skin reaction from vaccination; but at time of abortion it is reduced to almost neg. level. Reaction returns to sensitive level 7 days after abortion.	Aborts at 18 weeks. Foetus only 6-8 weeks old. *Pyometra* not produced before abortion but profuse discharge after.	Immune response consistently good during vaccination.
Group III								
A.17	*Not.*	Daughter of a vaccinated cow. Passive titre in blood during earliest months of life from ingestion of colostrum. Skin was also sensitised passively. Reaction normal for at least 6 months before infection.	At insemination.	Seen frequently from 3 weeks to after abortion.	Blood titre not above normal until 81st day when it became a low pos.; 13½:9; it rises to 15:9 after abortion. Blood titre returns to normal in 4 months.	Became positive at 4½ weeks (9-15). Became much less, sinking to negative level at time of pyometra. Returns to low positive (9-12) after abortion.	Aborts at about 2 months and 3 weeks. Foetus not found. *Pyometra* developed before abortion.	

TABLE II—*Contd.*

Heifers	*Vaccinated or not*	*Other previous treatment*	*How infected*	*Trichomonas seen*	*Titre of blood serum*	*Skin reaction*	*Result*	*Remarks*
B.17	*Not.*	Fed on whey at birth. Low passive titre. Skin response positive. Both reactions of short duration.	At insemination with strain "Manley."	Seen from 13th to 27th day, then not till 10 weeks when large numbers present. They disappear one day after abortion.	Not above normal until 10 weeks, when a low pos., 13:8½, then rises to high level at abortion. Normal 6 mths. after abortion.	Negative at 6 weeks, low positive (9-12) at 10 weeks. Increased reaction after abortion (8-13·5).	Aborts at 13 weeks, foetus 12 weeks old. Obvious pyometra not developed before abortion but discharge profuse after abortion.	The strain "Manley" is serologically different from Belfast. Tests carried out with homologous strain.
A.18	*Not.*	Low passive titre in early calfhood. Skin positive for short period.	At insemination with strain D.8 — a passage strain of "Belfast" cultivated from vagina of D.8.	Seen only at 4½ weeks in good numbers for 4 days. Then not again until seen at 7 months.	Not above normal level until 2 months and 1 week, then returns to normal at 5 months.	Becomes positive at 6 weeks, it fluctuates and is negative again at 3 months and 1 week.	Infection "takes," but calves normally at full term.	The infecting organism came from a passage strain.
A.11c, '43.	*Not.*	High passive titre from maternal colostrum. Mother highly vaccinated.	At insemination with "Belfast" strain.	Seen from the 3rd week for 25 days.	Above normal for 3 weeks during 3rd month. Then not signif. above normal 10½:9.	Positive for about 6 weeks during 3rd and 4th mth., then negative.	Infection "takes," but animal calves normally at full term.	
A.9c, '43.	*Not.*	High passive titre from maternal colostrum.	At insemination with "Belfast" strain.	Not seen during 9 months.	Not above normal.	Negative.	Infection seems *not* to have taken. Animal calves at full term.	

positive index. Sensitivity returned in a fortnight when the reaction was 8-13. In D.7 the skin reaction had become negative between the second and third doses of vaccine. The third injection did not alter the insensitive state but a good positive skin reaction was obtained as the subsequent infection developed. D.6 and D.8, which had reacted well to the first two vaccinations and produced very sensitive reactions in the skin test, showed no desensitisation after the injection of the third dose. There would seem to be a quantitative relation between skin sensitivity and the general state of the animal in regard to the distribution and fixation of antibody which would account for the variation in response to the injection of antigen into sensitised animals. Some animals are in a state in which the injection of further antigen causes desensitisation; others seem to have so high a degree of fixation of antibody that the dose of antigen introduced parenterally does not affect the sensitivity of the skin as regards the reaction to the skin test which remains positive. As will be shown later this mechanism also appears to act in acute infections.

In the vaccinated animals the blood tests and skin reactions were so much influenced by the vaccination that the effect of successful infections could not be traced. In most cases, however, especially where pyometra developed, the skin reaction became much less marked about the time of abortion, in some cases becoming negative. This occurred in D.6 and D.8 (pyometra cases) and also in one of the unvaccinated animals (B.17). It seems to be due to the absorption of a large amount of antigen resulting from the increase of the parasites in the uterus producing the same type of desensitising effect as did the intramuscular injection of the final dose of antigen in certain of the vaccination experiments.

In the non-vaccinated animals (A.17 and B.17) which became acutely infected, the effect of the progress of the infection on the blood titre and skin test yielded more information. In A.17, in spite of the acute character of the disease and the frequent appearance of Trichomonas in the vaginal secretion, the blood titre did not rise above the normal level until the 81st day when it was a low positive (13½:9), but after abortion it rose to 15:9. The skin test became positive at 32 days (9-15), was negative at the time of abortion and was a low positive (9-12) again after abortion. In B.17 the blood titre did not rise until the 70th day, when it became a low positive (13½:8), but after abortion reached a very high positive (19:8½). The skin test was negative at six weeks and became a low positive (9-12) at ten weeks. No drop was observed about the time of abortion and this may be connected with the absence of pyrometra in this animal. The blood titre in A.17 was at the normal level in four months after abortion and in B.17 in six months. Although these two infections were of the typical acute form it should be noted that the blood test would not have yielded a positive result until the 81st day in the case of A.18 and the 70th day in B.17. The

skin test was first positive at 32 days in A.18 and in B.17 on the 70th day. These points are emphasised, for while direct examination would have shown infection, the blood and skin reactions would not have given diagnostic evidence until a relatively late stage of the disease. Moreover, there is great variation in the period in the disease at which the serological and dermal reactions are developed. It is to be noted that in A.18 the skin test was positive before the blood titre was registered as above normal, but as has been shown when passive immune body is circulating in calves the specific antibody may be of so low a titre that it would be obscured in the blood tests in adults by the non-specific normal agglutinin. The degree of fixation in the skin appears to differ somewhat in different animals and does not seem to depend entirely on the *titre* of antibody although it is definitely governed by the presence of circulating antibody. The frequently positive skin tests and the rarely positive blood tests found in bulls is an illustration of this effect.

DISCUSSION

The salient feature of this set of experimental infections is that the circulating antibody produced in virgin heifers by the parenteral injection of the parasite does not protect them against infection. Thus the apparent self-limitation of the disease observed in the field must be due to some other mechanism or chain of circumstances. Another important aspect is the rapid disappearance of Trichomonas after abortion, although it must be reiterated that this does not always occur. This factor obviously reduces the value of abattoir examinations as a means of estimating the incidence of the disease in the cattle of any district. Attention is drawn to D.11 which was showing Trichomonas from the first infection at the subsequent oestrum when insemination was again unsuccessful. Infection was of the low-grade type which would have passed unnoticed under ordinary conditions. It is animals such as this which may infect the bull at a time when the presence of the disease is unsuspected.

The experiments throw some light on the manner of infection. The establishment of Trichomonas in the uterus of the heifer and the setting up of the acute disease seems to depend more on physiological conditions in the animal than on the parasite, for no great difference in the " virulence " of the strains used existed. If the passage strain of Belfast (D.8) and the Manley strain used for A.18 and B.17 are excluded, the infective organism was always the long cultivated Belfast strain, yet the severity of infections was very variable and nearly all the forms which occur naturally in the field cropped up in this small experimental series. Moreover, it is increasingly clear that *Tr. foetus* is a uterine parasite and that unless it establishes itself in that organ it does not seem to persist.

The details of sensitisation and desensitisation shown in this work will be reconsidered on another occasion but the question of diagnosis should be touched upon. All the methods described,

viz., direct examination, agglutination and skin test, are useful but none of them can be regarded as certain on any one examination. Direct examination may be negative for long periods in acutely infected animals and is mostly negative after abortion. The agglutination reaction and the skin test are strictly dependent on the production of circulating antibody in the serum as a response to infection. The skin reaction may not develop until shortly before abortion or may even be delayed until after that event; indeed, in an animal with an acute infection there may be negative periods due to temporary desensitisation. But it should be noted that during this phase the blood in the cases so far observed remains positive. Blood agglutinins, even in acute cases, develop late and, in certain instances, their presence may be of short duration.

Diagnosis depends on the intelligent appreciation of the nature of the history of sterility in a herd and the use of the three methods outlined above. A herd diagnosis should be obtained in most instances.

SUMMARY

An account is given of the experimental exposure of eleven virgin heifers to *Trichomonas foetus* infection by the introduction of the parasite into the uterine cervical canal at or four hours subsequent to artificial insemination in the middle of the heat period.

Four of the animals had previously been vaccinated intramuscularly with *Tr. foetus* and seven were controls.

Three of the four vaccinated heifers became infected and all three aborted. Of the seven controls, five became infected: two aborted and one failed to conceive; and two, although definitely infected, were slight cases and the pregnancies were not interrupted.

It is concluded that intramuscular vaccination, although producing a high titre of circulating antibody and a positive skin reaction, does not protect against infection with *Tr. foetus* introduced with undiluted semen at the time of insemination.

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STUDIES ON THE CLINICAL AND PARASITOLOGICAL ASPECTS OF CANINE DEMODECTIC MANGE

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INTRODUCTION

The parasitic nature of follicular mange was recorded by Tulk in 1843. Subsequent observations have been chiefly concerned with the morphology and systematic position of the mite and the pathology and clinical features of the disease with which it is associated. Nevertheless, several significant problems have not been adequately investigated. The finding of the mite in the skin of the normal dog has raised the question as to whether its presence in association with the lesions is not purely incidental and in this connection no attempt appears to have been made to determine the percentage of normal dogs which harbour the parasite, or to assess its significance both in the normal and in the diseased animal. In recent years *Demodex canis* has been found in the lymphatic glands of dogs suffering from follicular mange, but it has not been determined whether its presence in this situation is accidental, or represents a phase in the life-cycle of the parasite, either prior to or after, invasion of the skin. The small size of the parasite, its position in the skin and the difficulties associated with its transmission, are factors which have interfered considerably with an investigation of its life-cycle.

The present work comprises chiefly an enquiry into the parasitological aspects of the problem, but the clinical side has not been neglected, for it was felt that a better conception of the subject as a whole could be obtained only by exploring each of its various aspects.

As the literature is extensive it would be out of place in a paper of this kind to deal with it at length, but sufficient will be said to enable the work to be viewed in its proper perspective.

MATERIALS AND METHODS EMPLOYED IN THE INVESTIGATION

Demonstration of the Parasite in the Diseased Animal.—Skin lesions in dogs suffering from suspected follicular mange were scraped with a scalpel until the superficial layers of the skin had been removed and blood oozed from the scraped area. The material obtained was placed in 10 per cent. caustic potash, boiled until disintegration occurred and centrifugalised; the deposit was subsequently examined for the presence of mites.

Demonstration of the Parasite in the Normal Animal.—Small portions of skin, instead of scrapings, were obtained from the carcases of dogs shortly after death. The parasites were demonstrated by this method when the ordinary technique of scraping failed to reveal them.

Demonstration of the Parasite in the Lymphatic Glands.—Lymph glands removed from the diseased animal were incised and a search made for parasites in smears taken from the cut surface. In order to avoid contamination of the glands from the skin a separate set of instruments was used for

each stage in the operation, the skin incision being held open with forceps, so that the final dissection could be made without touching the cut surface. In those cases in which the mites in the glands were to be counted the entire gland was dissolved in caustic potash. Lymph glands removed from the normal animal were similarly treated and a search made for parasites in the deposit after centrifugalisation.

Method Employed in Counting Mites.—A known weight of skin from normal animals, generally 0·1 gramme, was dissolved in caustic potash, subsequently centrifugalised and the mites in the deposit counted. In animals suffering from follicular mange the number of mites in 0·1 gramme of skin was so great that a dilution technique had to be adopted by dissolving 0·1 gramme of skin in 0·9 c.c. of caustic potash and shaking vigorously to ensure an even distribution of the mites. Before the mites had time to settle, a sample of the fluid was placed in a helminth-ova-counting apparatus and a count made of the number of parasites in 0·05 c.c., the final result being expressed as mites per 0·1 gramme of skin.

Methods Employed in Isolating the Mites and in Transferring them to New Hosts.—Extensive scrapings were made from the skin of the affected animal into liquid paraffin. The choice of this somewhat unusual vehicle was based on a series of observations which showed that the mites could survive in it for nine days, and that the scrapings readily dispersed throughout the fluid on shaking, thereby facilitating the isolation of the parasites. After the scrapings had been shaken up, portions of the liquid paraffin were examined under a dissecting microscope and the adult mites removed by means of a fine mounted bristle to a clean drop of liquid paraffin, where they remained until arrangements for their transfer had been completed.

The animals used in the experiments on the life-cycle were young puppies varying in age from eight to ten weeks, although in a few instances puppies a fortnight old were used. The animal was anaesthetised and a small area of the skin of the spine was clipped, and marked with a tattooing needle. The mites were transferred one at a time to this area, which was kept under observation with the dissecting microscope, after which the puppy was allowed to recover and the marked area of skin removed after an appropriate interval of time.

In those cases where the object of the experiment was to transfer the disease in a clinical form, rather than to study the life-cycle of the parasite, a less tedious method was used in which a skin scraping of the affected animal in a small quantity of liquid paraffin was smeared on to the head of the experimental animal.

CLINICAL INVESTIGATION

It is generally believed that follicular mange occurs most frequently in young dogs and particularly in those animals in which the hair is short. Thus Wernicke and Stolte (1923) and Morris (1936) agree that the disease is most prevalent among dogs six to twelve months old. Morris found that 61 per cent. of cases were below the age of one year at the time of presentation, whereas only 17 per cent. were above the age of two years. The association of the disease with short-coated dogs, originally recorded by Cornevin (1868), has been more fully explored by Schindelka (1908) and by Morris, who have shown that more than 80 per cent. of cases occur

among dogs of this type. The predilection shown by the parasite for certain sites, such as the head, is well known, and has been commented on by a number of workers, notably by Friedberger and Frohner (1904), in connection with the uncomplicated (squamous) type of the condition. As far as the writer has been able to ascertain, no figures are available to indicate the frequency with which this, and other parts, are involved.

The present observations, made in the out-patient clinic of the University Veterinary Hospital, have enabled the author to confirm the work of previous observers on many of the clinical aspects of the disease.

A total of 51 cases was seen, the diagnosis, in every instance, being based on the demonstration of the parasite in scrapings taken from the affected areas. Forty-four (86 per cent.) were of the uncomplicated squamous type and seven (13·7 per cent.) had become complicated, probably as a result of secondary bacterial invasion.

Age is undoubtedly a significant factor in the incidence of the condition for 48 (94 per cent.) of the animals were under one year when first presented and the remaining three were approximately one and a half years old. Two of the latter group showed extensive involvement of the skin of long duration, and the presence of a staphylococcal infection. In the third case the extreme tip of the tail showed a lesion which had previously been disregarded on account of the small size of the area involved, but according to the owner had been in existence for about nine months. It is certain, therefore, that all the cases observed showed symptoms within the first year of life and that no diseased animal above this age was presented with a history of recent development of the condition.

The length of the hair would appear to have a bearing on the liability not only to infection, but also to invasion by bacteria. Thus, 76 per cent. of cases occurred in animals with short hair, the hair in the remaining twelve cases varying in length from that of a Highland terrier (*i.e.*, moderately long) to that of a retriever. The seven pustular cases occurred in this latter group.

As the clinical features of the disease have already been described in detail by many previous observers, it is not proposed to do more than make certain observations which appear to be of value for diagnosis. The parasite shows a predilection for certain sites. Thus the head was involved in 48 (94 per cent.) of the cases ; in 28 (55 per cent.) the lesions were confined to this area and occurred particularly around the eyes, in the temporal regions and at the sides of the muzzle. Such lesions, particularly those about the eyes, are often disregarded by the owner of the animal, as they may show little tendency to spread and the animal is eventually presented with a history of loss of hair from the affected part. As a rule the area is small, the exposed skin being slightly thickened and having a fine scaly appearance. In those cases which spread quickly the lesion may be up to 4 square inches in extent, the skin having approximately

the same appearance as in the purely localised type. In neglected cases the disease may spread outwards both from the head and from other foci which develop later, in particular the anterior aspects of the stifle and hock joints, so as to involve the greater part of the body surface. It is not known whether these cases would eventually have become pustular but one example of the generalised squamous type which has been in the writer's possession for a little over a year, and the skin of which has been repeatedly scraped, has shown no tendency to pustule formation.

Of the seven pustular cases observed, two were in animals one and a half years old, with a history of skin disease of long duration. Of the remaining five, three were approximately six months old when presented and the disease had not been noticed for more than a few months; in two, there was generalisation while in the third it was confined to the head. A bacteriological examination of these five cases revealed *Staphylococcus albus* in pure culture in four and associated with *Staphylococcus aureus* in the fifth.

Two of these pustular cases are worthy of special mention. The animals were first seen about a fortnight after the onset of symptoms, by which time the whole of the head and under aspect of the neck was completely denuded of hair, the underlying skin being hyperaemic, considerably thickened and velvety to the touch; the hair follicles stood out on its surface in the form of tiny prominences. Large numbers of pustules were present, particularly towards the margins of the affected areas. Examination of scrapings revealed large numbers of mites, together with their ova.

It would appear from the results of these observations that the onset of bacterial (secondary) invasion may occur simultaneously with, or follow closely upon, infestation by the acari, but the number of cases observed is too small to warrant any definite conclusions.

Although, in the majority of instances, the clinical lesions are fairly typical, confirmation of the diagnosis can be made only on the observation of large numbers of mites in scrapings from the affected parts. Less reliance can be placed on the finding of small numbers of parasites, for as will be shown later, 10 per cent. of clinically normal dogs harbour the acari in the skin. The significance of a few, *i.e.*, one or two per coverslip preparation, must be considered in conjunction with the age of the animal and with the type and distribution of the lesions.

LABORATORY INVESTIGATION

The Presence and Significance of the Parasite in the Skin of (a) the Clinically Normal Dog and (b) the Dog Showing Lesions

While references are frequently made to the occurrence of *Demodex canis* in the skin of normal dogs, such statements, so far as the writer is aware, have never been supported by numerical data. The view that the parasite may be present in normal skin was first suggested by Martemucci (1866) who assumed that it occurred in

all dogs, and later by Wernicke and Stolte (1923), and Vitzthum (1925). These assumptions were confirmed by Cameron (1925), who stated that he had found the parasite in apparently normal dogs. Horneck (1935), asserted that follicular mange exists in two forms, one in which the presence of the parasite in the skin is not associated with the production of lesions, and the other in which typical lesions develop.

In order to determine the incidence of the parasite in normal animals, 91 clinically normal dogs were examined and nine (approximately 10 per cent.) were found to be harbouring the parasite. In each of these animals the mites were found in the eyelids, while in five they were also found in the skin of the nose. In no case did they occur on the nose alone. Although the samples of skin were taken from the predilection sites, only a small portion from each animal could be examined, so that the percentage of normal dogs which harbour the parasite must be greater than that recorded here.

Having shown that the parasite occurs in the skin of the normal animal, an attempt was made to assess the significance of this finding by means of exact counts. In the normal animals the actual number of mites was counted in 0·1 gramme of skin, but in animals showing lesions the numbers in this weight were so great that the dilution technique previously referred to, was adopted, and the final figures estimated on the diluted sample.

TABLE I

SHOWING THE NUMBER OF PARASITES PRESENT IN 0·1 GRAMME OF SKIN, BOTH IN NORMAL ANIMALS AND IN ANIMALS SHOWING LESIONS OF THE DISEASE

Clinically normal animals		*Animals showing lesions*	
Case No.	*Total mites*	*Case No.*	*Total mites*
28	42	23	3,580
34	1,120	30	7,160
66	7	50	2,910
67	3	51	8,520
71	24	52	5,990
		53	399

The results as recorded in Table I, show that in all but a single instance, the number of mites present was much greater in animals showing lesions than in those that were clinically normal. The exception (Case 34) was an aged female and the absence of clinical lesions in this animal might be ascribed to the considerable degree of immunity to the effects of the parasite shown by animals above the age of one year. It is possible that a similar degree of infestation in a young animal would have provoked the disease in a clinical form. The 2,910 mites in Case 50 represent the lowest total number recorded on any occasion from this individual. Case 53, in which only 399 mites were present, resolved spontaneously, although at the time of observation a small lesion was present on the head.

It has been shown that while the number of parasites which occur in the normal dog is usually small, it may be considerably larger in the aged animal. Although little reliance can be placed on a single observation it is suggested that such normal " carrier " animals may play an important part in the epizootiology of the condition.

The Presence and Significance of the Parasite in the Lymphatic Glands

The presence of *D. canis* in the lymphatic glands of dogs suffering from follicular mange, was first recorded by Canepa and da Grana in 1941, who noted an increase in size of the superficial lymph nodes, particularly in cases of the pustular type, and recorded the isolation of the parasite from the submaxillary, retropharyngeal, prescapular, inguinal, popliteal, mediastinal and mesenteric glands. They suggested that when the parasite seemed to disappear temporarily from the lesions, the lymphatic glands might constitute a reservoir of infection from which the acarus might subsequently regain the skin. The potential importance of these observations and the lack of confirmation of the findings, led the writer to examine the lymph glands of diseased and clinically normal animals for the presence of *D. canis*.

The observations on normal animals are based on the examination of the submaxillary glands which receive the lymph from the head and are thus most likely to be infected. Of 91 clinically normal dogs, two (2·2 per cent.) harboured the parasite in the glands as well as in the skin. The parasite has not been found in the glands of the normal animal in the absence of skin infestation, but, in seven instances, has been recovered from the skin in the absence of demonstrable glandular infection.

The parasite was not seen in the prescapular and internal iliac glands of 24 normal dogs. In six animals with clinical signs of disease, mites were demonstrated as follows: on five occasions in the submaxillary, four times in the prescapular, three in the internal iliac, and on one occasion in the portal and mesenteric glands. In a further three cases, in which the lesions were small and confined to the head, glandular infection was not discovered.

In most instances examination was made for living mites, but live specimens were observed only on one occasion. Too much reliance, however, must not be placed on this statement as viable parasites not infrequently remain quiescent, and would therefore here be classified as dead on the only criterion of life open to the observer, *i.e.*, that of definite movement.

The results of such an examination being in doubt, it appeared important to determine whether infestation of the glands was primary or secondary to the skin infestation, and if the latter, whether the passage of the mites to the glands was an active movement on their part, or whether they were passively carried there in the blood or lymph stream. An answer to this part of the problem would furnish

evidence as to the significance of the lymph gland infestation, both in relation to the clinical aspects of the disease, and in relation to the bionomics of the parasite.

If the results so far recorded are examined, it will be seen that in both clinically normal and clinically diseased dogs parasites have not been found in the lymph glands in the absence of skin infestation. The fact that the mites may be present in the skin only would indicate that the infestation of the glands is secondary to the skin infestation. This view was supported by the results of transmission experiments, showing that the mites were able to exist in the skin of the experimental animal for a period of one month without producing infection in the associated lymphatic glands.

In view of this finding it became necessary to determine the route or routes by which the parasites reached the lymphatic glands. A count was therefore made of the number of mites present in certain of the lymphatic glands of two animals to discover whether the parasites showed any degree of selectivity in respect of particular glands. The results were as follows :—

Case 1. Submaxillary, 20 ; prescapular, 614 ; internal iliac, 107.

Case 2. Submaxillary, 36 ; prescapular, 266 ; internal iliac, 67.

In both, the prescapular gland was the most heavily infested, and the submaxillary glands, by comparison, showed few mites. The latter receive lymph from the relatively small area of the head whereas the area drained by the prescapular gland is large, for it receives the lymph from the dorsal and lateral aspects of the neck and from the upper parts of the head as far forward as the lips. The internal iliac glands occupy an intermediate position, receiving lymph from the pelvis and indirectly from the posterior part of the abdominal wall, and from the hind limbs. Attention is drawn to the fact that, while the area drained by the internal iliac glands is probably as large as that drained by the prescapular glands, the hind limbs of both the animals mentioned showed no lesions of the disease. The results, therefore, suggest that the degree of infestation of the lymph glands depends upon the extent of the skin area which they drain, and also that the parasites travel to the glands by way of the lymph or blood streams, for if they passed directly through the tissues, a selective action in respect of certain glands would not be expected. Another factor which is possibly of some importance in determining the degree of infestation of the lymphatic glands, is the number of mites present in a unit weight of skin. Thus a clinical case which had a total of 399 mites in 0·1 gramme of skin removed from the head showed no parasites in its submaxillary gland, despite the fact that the lesion involved an area of 4 square inches. On the other hand, parasites were found in the submaxillary glands of another dog showing no lesions, in which the mite count per unit weight of skin from the nose was 1,120. When the counts from the skin were higher the lymphatic glands were almost invariably involved, although in these cases the skin lesions were generally more extensive. Two

factors, size of area infested and degree of infestation, together with a possible third factor, *viz.*, duration of infection, are probably interdependent, and it is not possible to say which is the most important in determining the extent of invasion of the lymphatic glands.

While these results indicate that the parasite reaches the lymphatic glands *via* the blood or lymph stream, it remained to be shown whether the mites penetrate into the vessels in fulfilment of a phase in their life-cycle, or whether their presence in the lymphatic glands was accidental. A series of experiments was undertaken in two separate animals, in which the total mite population and the proportions of the various stages in the lymphatic glands were compared with similar counts obtained from 0·1 gramme of skin removed from the heads. The results of these experiments are shown below in Table II.

TABLE II

SHOWING, IN TWO DOGS, THE TOTAL MITE POPULATION AND THE PROPORTIONS OF ITS VARIOUS STAGES IN 0·1 GRAMME OF SKIN AND IN THE SUBMAXILLARY, PRESCAPULAR AND INTERNAL ILIAC LYMPHATIC GLANDS

Animal	*Tissue examined*	*Total mite population*	*Percentages of various stages*		
			Adults	*Nymphs*	*Larvae*
A	Skin	3,590	88·3	6·4	5·3
	Submaxillary gland	36	77·8	11·0	11·0
	Prescapular gland	266	88·7	6·3	5·9
	Internal iliac gland	67	89·5	5·9	4·5
B	Skin	7,160	59·8	21·2	19·0
	Submaxillary gland	20	85·0	5·0	10·0
	Prescapular gland	614	63·2	21·8	15·0
	Internal iliac gland	107	58·7	22·4	18·7

It will be seen that despite the high mite population of the skin, the total counts in the submaxillary glands were only 20 and 36 respectively, *i.e.*, 1·0 per cent. in the one case and 0·3 per cent. in the other, of the population in 0·1 gramme of skin. This weight of skin measures approximately $\frac{1}{4}$ inch square, so that the number of parasites in the total area draining to the submaxillary gland and the number theoretically able to reach them is far larger than that which actually does so. It can further be seen that the proportions of the various developmental stages in the lymphatic glands are approximately equal to those in the skin. While it would seem that the submaxillary glands do not run true to type, it may be pointed out that the number of mites occurring in these glands is comparatively small, so that the addition or subtraction of any mite of any stage would have a correspondingly large effect on the percentage. On

account of the larger number of parasites occurring in the prescapular and internal iliac glands, this error in the case of these glands is considerably reduced.

It would appear, therefore, that the entry of the mites into the lymphatic system is accidental, and that they are passively carried to the lymphatic glands, for if the movement were an active one, or if it constituted a phase in the life-cycle, it would be reasonable to expect the number of mites which succeeded in reaching the glands to be much larger. If to each stage in the life-cycle is accorded an equal chance of reaching the lymphatic glands, then the percentages of the various stages present in the glands would be approximately equal to those in the skin. The figures in Table II tend to confirm this hypothesis.

Transmission of the Parasite from Diseased to Healthy Dogs

Since the time of Weiss (1860) many workers have attempted to transmit follicular mange in a clinical form, but in only four instances has success been claimed. Haubner (1859) and Cornevin (1868), recorded transmission by the inoculation of pus containing the parasites to healthy dogs. The lesions disappeared after a short time—in Cornevin's case after eight days. Guinard (1890) and Lemke (1897) also claimed to have transmitted the disease in a clinical form.

The difficulty in transmitting the parasite has given rise to the view that the mites can only multiply to an extent sufficient to cause lesions when some special susceptibility exists in the animal to which they have gained access. Canine distemper, youth and concurrent skin disease are considered to be important predisposing factors (Lafosse, 1861 ; Lifka ; Reichart, 1924). Some observers (Wernicke and Stolte, 1923 ; Morris, 1936) consider that the time of change from deciduous to permanent dentition plays an important part. Horneck (1935) has attempted to classify these factors into those which predispose to infection and those which influence the spread of the disease on the infected animal.

While the writer fully realised the difficulties associated with the transmission of follicular mange, it appeared to him not unlikely that the parasites themselves might be transferred with relative ease and that it might be possible to investigate the life-cycle by applying the parasites to the skin and removing the portions of skin to which they had been transferred, after varying intervals ; a procedure which had already been employed with success in the case of *Notoedres* (Gordon *et al.*, 1943).

Two experimental methods have been investigated, *viz.*, skin transmission and oral transmission.

Skin Transmission.—Before presenting the results obtained, certain points should be made clear.

Since only 10 per cent. of clinically normal dogs have been found to harbour the parasite and in view of the difficulty in demonstrating

the mites in such animals other than by the complete removal and examination of whole portions of skin, a procedure which would have interfered with the subsequent transfer of the parasites to the area selected for this purpose, the puppies used in this series of experiments, except in one instance, were not examined prior to being placed under experiment. In order to limit the error which might have resulted, as well as for technical reasons, the predilection sites were avoided in making the transfer, the mites generally being applied to a shaved area of the back. After death, none of the puppies was found to be harbouring mites in the skin of the predilection sites and they were therefore regarded as being free from infection prior to experiment. In order to recover the mites which had been transferred to the experimental animal, the skin prior to its removal was rubbed with a swab soaked in spirit, a procedure which would have removed any parasites which might have been present upon its surface. It will be seen later that all stages in the life-cycle of the mites were recovered six weeks after their transfer to the uninfected animal, suggesting that they had actually entered the follicles. In view of the small size of the mites and the small numbers present, it was not possible to devise a technique by which one could demonstrate whether they were alive or dead, except in those cases where the mites had undergone reproduction in the course of the observation period.

Clinical lesions failed to develop in 14 dogs which were observed for periods varying from three to 47 days following the transfer of the parasites. In contrast to this failure to provoke the disease in a clinical form, it was comparatively easy to transmit the mites themselves, although in most instances they failed to produce ova during the observation period. In two out of four cases, both of which were puppies a fortnight old at the time the first transfer was made, the parasites had failed to establish themselves in the skin at the time of *post-mortem* examination, 39 days and 41 days later. The remaining two puppies, aged a fortnight and two months respectively, were exposed to infection by means of skin scrapings on four occasions at intervals of four days. Parasites were found in the area of skin to which they had been transferred, 31 days and 47 days respectively after the last transfer. In both cases, all stages of the mite were present, although the total population in 0·1 gramme of skin was very small; seven in the case of the younger, and 56 in the case of the older puppy. Having regard to what has previously been said on the infestation of the lymphatic glands, it is of interest to note that in neither case were parasites found in the submaxillary glands.

The remaining direct transmission experiments were undertaken in an attempt to determine the life-cycle of the parasite. Ten puppies eight to ten weeks old were used, from 60 to 100 mites being transferred to a marked area of the skin of the back. Only adult mites were used, for as shown above the number of immature forms available in skin scrapings is very small. Although larvae were

occasionally encountered, the writer was unable to remove them from the slide for transference to dogs. It was, of course, appreciated at the outset that it would not be possible to say whether the adults, later found, were those which had been originally transferred, or whether they belonged to the second generation.

The results of this series of observations are shown below in Table III.

TABLE III

SHOWING, IN A SERIES OF 10 PUPPIES, 8 TO 10 WEEKS OLD, THE RESULTS OF THE EXAMINATION OF AREAS OF SKIN TO WHICH 60 TO 100 ADULT MITES HAD BEEN TRANSFERRED 3 TO 19 DAYS PREVIOUSLY

Animal	*Duration of experiment in days*	*Results of examination of skin area at autopsy*			
1	3	4 adults			
2	3	4 „			
3	3	16 „			
4	6	6 „	9 ova		
5	7	4 „			
6	8	4 „			
7	10	5 „	2 larvae	3 ova	
8	12	14 „			
9	13	5 „			
10	19	14 „	4 nymphs	6 larvae	3ova

It can be seen that mites were recovered at periods ranging from three to 19 days, although in the majority of instances they had remained quiescent and had failed to oviposit. This failure to reproduce may have been due to the fact that the females had not been fertilised prior to their entry into the skin, but the large numbers which were transferred would appear to negative this explanation. It is also conceivable that, in most of the animals, the skin was unfavourable to the reproduction of the parasite, an explanation which would accord well with the generally accepted view that the mites can establish themselves only when some predisposition exists in the animal to which they have been transferred.

The data obtained from those cases in which the mites reproduced in the skin, show that the incubation period of the eggs is a little over six days, for one of the ova recovered on that day contained a fully developed larva. On the tenth day, moulting forms, larva to first nymph, were present. The results recorded on the 19th day are difficult to interpret for they may indicate, either that the life-cycle was nearing completion, or that it had been completed and some of the adults found were of the second generation. It is possible to state, even on the basis of so few results, that the life-cycle up to the second nymphal stage can be completed in 19 days, although it is conceded that this may not be the minimum period. It would appear that until a condition of susceptibility to the attack of the mite can be produced at will, the minimum duration of the life-cycle is likely to remain undetermined.

Oral Transmission.—The finding of mites in lymphatic glands suggested that they might reach the skin *via* the intestinal canal and lymphatic system, and that they might spread from one area to another, or be transferred to a fresh host by this route. Although the evidence already provided indicates that this is unlikely, the suggestion received some support from an observation that the mites were able to survive immersion in 0·6 per cent. hydrochloric acid and 0·5 per cent. pepsin, for a period of 22 hours.

Scrapings were taken from a case of follicular mange directly into the hydrochloric acid-pepsin solution in a test tube. The mixture was shaken and several drops placed between slide and coverslip and sealed round the edges with wax. The mites were examined at hourly intervals from noon until 6 p.m., left on the bench overnight and hourly examinations resumed the following morning. The mites were still alive after 22 hours, when the experiment was abandoned.

Three puppies were fed five times during the course of a week, on pieces of skin approximately $\frac{1}{2}$ inch by 2 inches, removed from cases of follicular mange. Although the number of parasites was not counted each puppy must have received several hundred thousands of mites. The puppies were destroyed at the end of a month. Examination of the submaxillary, prescapular, mesenteric, portal and internal iliac lymphatic glands and of the skin of the predilection sites, failed to reveal a parasite. In another experiment, a puppy was fed an entire prescapular gland (the corresponding gland from the opposite side of the body contained 614 mites). Subsequent examination of the lymphatic glands and of the skin was negative. It would appear, therefore, that within the period of observation the mites had not reached the skin by travelling *via* the intestine and lymphatic glands.

DISCUSSION

Demodectic mange has always been regarded as a most refractory skin disease of the dog, since prognosis is difficult and no form of treatment will bring about a certain cure.

From the clinical aspect certain general principles, likely to be of value in diagnosis, have been formulated. The present investigation confirms the findings of previous workers that the disease occurs principally among short-coated dogs below the age of one year. It is true that Morris records 38 per cent. of his cases in animals above the age of one year, but this observation would appear to refer to the age of the animal at the time of presentation and little account appears to have been taken of the time at which symptoms first became manifest. As a general rule Morris's cases involved a much greater area of the body than those seen by the present writer. He notes that 49 per cent. of his cases involved more than 25 per cent. of the body surface, whereas 68 per cent. of those recorded in this paper were of the localised type. The writer disagrees with St. Cyr (1876) that the parasites show no preference for certain areas of the

body. In the majority the head is involved to a greater or lesser degree, the lesions frequently being confined to this area and showing little or no tendency to spread.

An examination of a number of clinically normal dogs has revealed a " carrier " population which may play a part in the spread of the condition. Probably the chief offender is the " carrier " bitch, which as a result of close and prolonged contact, transmits the disease to her puppies during the suckling period. Although the puppies may harbour the parasite they may show no signs of disease until a later date, when the development of a condition of susceptibility allows the mites to become invasive and transforms a latent into a clinical infection.

In recent years attention has been focused on a new and entirely different aspect of follicular mange, an aspect which was first studied by Canepa and da Grana, and which has been treated at some length during the course of the present investigation. It appeared at first sight that the presence of *Demodex canis* in the lymphatic glands of the dog might be of importance both from the clinical standpoint in constituting a reservoir of infection, and probably also from the point of view of the life-cycle of the parasite. From an examination of a considerable number of normal animals and of a smaller number of infested ones, and from a comparison of the degree of infestation in the lymphatic glands in relation to that of the skin, it would appear that the movement of the mites to the lymphatic glands is of a passive nature, a view which has received support from the fact that the constitution of the population in the glands is approximately equal to that in the skin. Whilst it is conceded that the evidence is not extensive, it is considered sufficient to warrant the conclusion that the mites are unable subsequently to regain the skin from the lymphatic glands.

A number of workers have shown that follicular mange is not readily transmitted to healthy dogs even when they are housed for prolonged periods with diseased animals ; nor is it possible, except in a minority of cases, to transmit the infection by transferring the mites in pus taken from cases of the disease. While the writer has failed to evoke the disease in a clinical form, he has, however, been able to transmit the parasite, although in the absence of a predisposition on the part of the animal to which the parasites were transferred, the latter have never multiplied to an extent sufficient to produce lesions.

SUMMARY

Observations on 51 cases of canine demodectic mange have shown that in every instance the onset of symptoms occurred at some time during the first year of life.

Forty-five (86 per cent.) were of the uncomplicated, squamous type and seven (13·7 per cent.) had become complicated as a result of bacterial invasion.

Thirty-nine (76 per cent.) were in animals with short hair. Of the remaining twelve, which occurred in long-coated animals, seven had become complicated.

The lesions were confined to the head in 28 (55 per cent.).

It has been shown that 10 per cent. of dogs may harbour the parasite without showing lesions and that the number of mites per unit weight of skin in these animals is very much smaller than in diseased animals of a comparable age.

It is suggested that such " carrier " animals may be important sources of infection.

Demodex canis has been demonstrated in the lymphatic glands of both clinically normal and of clinically diseased dogs, but never in the absence of a skin infestation.

The evidence indicated that the glandular infestation is secondary to that of the skin, and that the mites are carried passively to the glands.

It is suggested that the mites neither reproduce in the glands, nor are they able subsequently to regain the skin.

While it has not been possible to transmit follicular mange in a clinical form, the transmission of parasites to uninfected animals by direct skin transfer was not difficult.

In general such animals show no symptoms of the disease and it is suggested that lesions only develop when the presence of some special susceptibility allows the parasites to become invasive.

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THE PROPHYLAXIS OF BOVINE BLACKQUARTER

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INTRODUCTION

Although owners of cattle in general appear to be satisfied with the results obtained in immunisation against blackquarter, the present position has several undesirable features, the most important being the continued use of agents of doubtful value and the absence of potency tests. The common agents used in blackquarter prophylaxis are so well known that only a brief description, sufficient to clarify the nomenclature, is necessary for the purposes of this paper. Pellet or pilule vaccine, one of the earliest forms of vaccine, is still widely employed in many parts of the world. It is prepared by heat attenuation of the organism in muscle from infected cattle and is administered by implantation under the skin with a specially designed injector. Natural aggressin or tissue filtrate was widely used before the introduction of culture vaccines and still retains its popularity with some practitioners. It is obtained by filtration of the tissue exudate from infected cattle. Artificial aggressin is a filtrate of a culture of *Cl. chauvoei* and is frequently used by practitioners who are averse to using material containing organisms. Formalinised whole culture is prepared by the addition of formalin to a culture of *Cl. chauvoei.*

In the absence of a standard test for potency it has been impossible to grade the various blackquarter vaccines according to their antigenic values, and the practitioner, in making his selection, has been guided largely by secondary considerations such as convenience of administration.

Before undertaking this work the writer had reason to believe that if a satisfactory test of the value of these vaccines could be applied it would be possible to recommend the limitation of their range to one or two approved types. It was realised, however, that the pilule method was so firmly established in many parts of the British Isles that the strongest recommendation based on potency might fail to discourage its use, and for this reason it might be desirable to select two vaccines for general use, *viz.*, one in liquid and one in pilule form. In view of many serious disadvantages in the existing pilule vaccines it was felt that if their use was to be continued it was desirable to alter their composition, and an attempt was therefore made to develop a satisfactory vaccine in pilule form.

The work recorded here thus covers three phases of blackquarter immunisation, *viz.*, (1) the elaboration of a satisfactory test for potency of blackquarter vaccines; (2) the application of this test to existing vaccines and to a new form of pellet vaccine in order to survey their antigenic values; and (3) the development of a new type of vaccine in the form of a pellet.

TECHNIQUE AND MATERIAL

The strain of *Cl. chauvoei* used was VR136, isolated from a calf in 1938 and since kept in desiccated muscle, with two passages in cattle. Its virulence at the time of this work can be approximately defined by stating that when a 48-hour broth culture is injected intramuscularly in 350 gramme guinea-pigs in doses of 0·1 ml., it causes death in about 50 per cent. of the animals. There has been no appreciable loss of virulence over a period of seven years.

Routine laboratory cultivation has been carried out in a 4 per cent. solution of peptone (prepared at the Institute) in tap water containing 0·5 per cent. sodium chloride, heated in a steam jacketed pan to 90° C. at a *p*H of 8·5 and the precipitate removed by filtration. The broth is then put into tubes or flasks, autoclaved at 15 pounds pressure for 20 minutes, and 0·5 per cent. of glucose added.

The spore suspension was made once only, the same suspension having been in general use for two years. It was prepared from a 2·5 litre quantity of peptone broth culture, which had been incubated for 48 hours and allowed to age at room temperature for two weeks. The organisms were removed by decanting and centrifuging, and were then washed three times in physiological saline. The density of the final suspension was adjusted by opacity to 10,000 million organisms per c.c.

In order to obtain uniform groups, both experimental and control guinea-pigs were weighed before the first injection of vaccine and in some tests before the infective dose was administered. Guinea-pigs of 350 grammes or more were used. All except those which survived for more than five days after the injection of infective material were examined *post mortem*. Death was presumed to have been due to infection with *Cl. chauvoei* when macroscopic lesions and liver impression smears were typical, but a negative result was accepted only if anaerobic cultures from the heart blood were sterile.

POTENCY TESTS

The demonstration of immunity resulting from injection of culture filtrate and of formalinised culture vaccines, has been achieved without much difficulty. Thus, McEwen (1926) studied the immunity conferred on guinea-pigs by injections of non-toxic filtrates and of formalinised culture by injecting the guinea-pigs with virulent culture. Viljoen (1926) in a comprehensive study of blackquarter vaccines, also used intramuscular injections of culture to test the immunity conferred on sheep. Henderson (1932) and Roberts (1933) used graded quantities of washed organisms, activated with a constant quantity of calcium chloride solution, to test the immunity of guinea-pigs treated with the " O " antigen of ovine strains of *Cl. chauvoei*. Goss and Scott (1918) proposed a test based on the power of an " aggressin " to activate a sub-lethal dose of culture administered to guinea-pigs previously protected with serum, and Scott (1932) gives a further account of this test. This method is of course designed

to give a measurement of " aggressin " activity and assumes that this activity is equivalent to potency.

The results of preliminary work had suggested that a satisfactory standard test could be based on the principle of immunising guinea-pigs with a given preparation, and testing the animals' resistance by injecting them subcutaneously with a suspension of *Cl. chauvoei* spores activated with calcium chloride. From a theoretical point of view it appeared to us that when spores and an activator are injected, the activator, which presumably causes the initial necrosis and allows the spores to germinate and propagate, is more likely to be the deciding factor in infection than the spores themselves.

TABLE I

EFFECT OF VARYING THE AMOUNT OF ACTIVATOR IN THE PRESENCE OF A FIXED DOSE (0·1 ML.) OF SPORE SUSPENSION

Number of guinea-pigs used	*$CaCl_2$ (5 per cent. solution)*	*Result*
10	0·5 ml.	++++++++++
10	0·25 ml.	+++++++++LL
10	0·125 ml.	++LLLLLLLLL
10	0·0625 ml.	LLLLLLLLLL

+ = Died within 30 hours. L = Survived beyond 5th day.

This view was confirmed by the results of experiments. When a fixed quantity of spores was injected with calcium chloride it was found that the death-rate increased precipitously as the amount of activator was increased (Table I); further, the smallest quantity of activator which caused death in all pigs of the group gave the same result when the amount of spore suspension was reduced 1,000 times, from 0·1 ml. to 0·0001 ml. It was concluded that a test for immunity based on this method of infection would be unaffected by variations in the spore suspension. Thus, the quantity of spore suspension could be arbitrary and the calcium chloride solution looked upon as the " toxin " to be varied according to the severity desired in the test. It has not been possible to carry out enough tests to demonstrate the extent of the variation which occurs with this method of infection but an impression has been gained that, because of the excessive number of spores used, the individual variation in the susceptibility of guinea-pigs to infection with *Cl. chauvoei* is to a great extent eliminated.

In applying this method of infection to test the immunity of guinea-pigs it was thought that the severity of the test dose would have to be varied in the light of experience. It was found, however, that some vaccines conferred resistance of a high order and it was therefore decided to work tentatively with an amount of activator which caused the death of ten out of ten control guinea-pigs. We have had no evidence to suggest that this dose is too severe, but in view of the possibility that occasional guinea-pigs may have a high degree of resistance, it is considered that in framing a test for

immunity it would be sufficient to rule that the test dose should kill 80 per cent. or more of the control guinea-pigs within five days. The routine method finally adopted is as follows. The vaccine under test is injected subcutaneously into groups of not less than ten guinea-pigs. The first dose of vaccine is 2·5 ml. and the second, given ten days later, is 5 ml. After a further interval of ten days the immunised guinea-pigs and a control group which has been segregated from the commencement of the experiment, are injected intramuscularly with 0·1 ml. of spore suspension and sufficient 5 per cent. solution of calcium chloride to cause the death of 80 per cent. of normal guinea-pigs, as determined by a preliminary titration.

It is desirable that the results of laboratory tests should be confirmed by field trials, but the low and irregular incidence of blackquarter makes it unlikely that this ideal can ever be achieved with blackquarter vaccines. The value of the test must be assessed, therefore, on results obtained in the laboratory, and the ensuing section of this work is designed to enable such an assessment to be made.

SURVEY OF THE POTENCY OF BLACKQUARTER VACCINES

Four tests in all were carried out upon different groups of vaccines, but for convenience of recording, the results have been combined in one table (Table II). The vaccines tested were purchased in the open market and by withdrawing specimens of material, already passed for issue, at our own laboratories. Tissue filtrate No. 1, and pellet vaccine No. 3, were prepared in the laboratory for reasons explained later. The amount of calcium chloride used was 0·5 ml. of a 5 per cent. solution.

Results.—The most remarkable feature of these tests is the manner in which the vaccines fall into two well-defined groups, effective and ineffective. If it is stipulated that in order to pass the test, an antigen should protect 80 per cent. of guinea-pigs, it will be noted that out of 20 vaccines tested, nine pass, and among the remaining eleven the highest survival rate is 15 per cent. Of the nine formalinised culture vaccines, all pass except two, one of which, as shown by smear examination, was not a culture of *Cl. chauvoei* (Table II). These formalinised culture vaccines represent the market products of six laboratories, and it appears reasonable to conclude, therefore, that the test is not unnecessarily stringent. Each of the five samples of culture filtrate was marketed by a different manufacturer. Four of them failed to satisfy the test and as three of these were obtained from laboratories which supplied satisfactory samples of formalinised culture vaccine it is concluded that it is harder to produce a good antigen in the form of filtrate than in the form of whole culture. The only sample of tissue filtrate which satisfied the test was prepared in a laboratory experiment. We consider that " aggressin " or tissue filtrate can constitute a good antigen, but that owing to filtration

TABLE II

APPLICATION OF THE TEST TO MARKET AND LABORATORY SAMPLES OF BLACKLEG PROPHYLACTIC AGENTS

Nature of vaccine		*Average weight of G.P.s*			*G.P.s in test*	*G.P.s surviving*	*Percentage survival*
		Before 1st vaccination	*Before 2nd vaccination*	*Before infection*			
Formalinised culture	(1)	—	—	—	9	9	100
" "	(2)	447	386	335	12	10	83
" "	(3)	—	—	—	10	8	80
" "	(4)	488	—	503	11	11	100
" "	(5)	427	492	503	14	12	86
" "	(6)	434	481	486	12	12	100
" "	(7)	430	490	488	14	12	86
Culture filtrate ...	(1)	468	510	525	13	11	85
Tissue " ...	(1)	—	—	—	10	8	80
Formalinised culture	(7)*	443	499	511	13	0	0
Formalinised culture of *Cl. septique* and *Cl. chauvoei*		443	483	480	14	1	7
Culture filtrate ...	(2)	—	—	—	10	0	0
" " ...	(3)	428	450	472	13	2	15
" " ...	(4)	438	500	479	14	1	7
" " ...	(5)	455	443	451	13	1	8
Tissue " ...	(2)	—	—	—	10	1	10
" " ...	(3)	436	435	444	13	1	8
Pellet vaccine ...	(1)	—	—	—	9	0	0
" " ...	(2)	457	491	482	14	0	0
" " ...	(3)	—	—	—	15	0	0
Controls (4 groups of 10, 12, 14 and 15 guinea-pigs respectively) ...		—	—	—	51	1	2

*Not *Cl. chauvoei* on evidence of morphology.

Each guinea-pig injected with 2·5 c.c. and 5 c.c. of vaccine or one and two pellets with a nine-day interval. Nine days later the guinea-pigs, together with a corresponding control group, were injected with 0·1 c.c. of a suspension of *Cl. chauvoei* spores and 0·5 c.c. of a 5 per cent. solution of calcium chloride.

Twelve guinea-pigs were lost from intercurrent disease out of the total of 291 submitted to tests. The greatest number lost in one group was three, lost in the pellet vaccine (1) group.

difficulties it cannot be prepared satisfactorily under manufacturing conditions. All three pellet vaccines failed to show evidence of antigenicity, and not one guinea-pig survived in three groups tested. The laboratory sample (Sample 3) was prepared with meticulous attention to temperature and time of storage at each stage in its preparation during which no extraneous organisms appeared. *Cl. chauvoei* in pure culture was isolated from the calf muscle before and after drying, from the powder after attenuation and from the finished pellets. As the organisms isolated after "attenuation" were still pathogenic to guinea-pigs, the result obtained could not have been due to excessive attenuation. It is perhaps difficult to anticipate results in the field from a guinea-pig test on a living vaccine, and the results obtained here may not completely condemn

this form of prophylaxis ; nevertheless, it is obvious from them that pellet vaccines are unlikely to satisfy the guinea-pig test.

Both the guinea-pigs treated with formalinised culture (Sample 2), and their control group, lost weight during the same interval, but a satisfactory explanation was found in a deficiency in the food supply. Apart from this group, the animals maintained or gained weight during the vaccination period, and there seems to be no relation between the protective value of the agent and its effect on the weight curve. This appears to dispel any suggestion that an apparently harmless agent such as culture filtrate has an advantage over others. We consider that the test outlined above is useful and that laboratories adequately equipped and staffed should find no difficulty in preparing formalinised culture to satisfy the test. Types of vaccines which failed to pass could be justifiably discouraged in that they are less antigenic than other easily prepared materials.

We have considered the desirability of providing a standard antigen and suggest that possibly the new type of vaccine described below may serve as a dried standard.

THE PREPARATION OF A NEW TYPE OF PELLET VACCINE

To meet requirements in the field it might be impossible to prevent the use of pellet vaccines because they are convenient and their use has become a firmly established tradition in many areas. It has been shown here that a test which clearly demonstrated the antigenic potency of liquid vaccines, failed to reveal any degree of potency in existing pellet vaccines. These facts, together with a knowledge of the difficulties inherent in preparing the latter, emphasised the need for replacing them with a pellet vaccine of proved antigenicity.

The preparation and testing of a dry vaccine made by precipitating the proteins from formalinised cultures of *Cl. chauvoei*, drying the precipitate and combining it with organisms obtained by centrifuging the same culture is described below. Early in this investigation it was shown that some degree of immunity was conferred by such a vaccine. The first experiment, recorded in Table III, was carried out with the ammonium sulphate precipitate and organisms from a liver broth culture of *Cl. chauvoei* treated with formalin. It was found that each pilule as made represented 2 c.c. of the original culture, and it was therefore compared with that amount of the latter in respect of its immunising value for guinea-pigs. One month after having been immunised subcutaneously the guinea-pigs in these two groups, together with nine controls, were each injected intramuscularly with 0·1 c.c. of a 48-hour broth culture of *Cl. chauvoei*.

While this experiment showed that protection was conferred by the dry vaccine, several incidental defects were detected. It was realised firstly that the method of testing was inadequate and further

TABLE III

COMPARISON OF ANTIGENIC ACTIVITY OF NEW PILULE VACCINE AND FORMALINISED CULTURE

No. of guinea-pigs used	*Immunising agent*		*Infection*	*Result*	*Percentage survival*
12	1 pilule	s.c.	0·1 c.c. broth culture	L L L L L L L L L L + –	91
12	2 c.c. of formalinised culture	„	0·1 „ „	L L L L L L L L – – – –	100
9	—		0·1 „ „	L L + + + + + + + +	22

L = Living 5 days after infection. + = Died within 5 days. –– = Died of intercurrent disease before receiving culture.

TABLE IV

ANTIGENIC TESTS ON THE CULTURAL PILULES AND ON THE FORMALIN KILLED CULTURES FROM WHICH THEY WERE PREPARED

Antigen used	*1st injection*	*2nd injection (after 10 days)*	*Result of immunity test (0·1 c.c. of spore suspension and 0·5 c.c. $CaCl_2$ anhydrous) in 5 per cent. solution (after 10 days)*	*Percentage survival*	*Average weight of guinea-pigs*	
					Before vaccination	*After vaccination*
Experiment 1						
Formalinised culture... Sample 1	2·5 ml.	5 ml.	L L L L L L L + + +	70	—	—
Pilule vaccine (cultural) Sample 1	1 pilule	2 pilules	L L L L L L L L +	80	—	—
Controls	—	—	+ + + + + + + + + + + +	0	—	—
Experiment 2						
Formalinised culture... Sample 2	2·5 ml.	5 ml.	L L L L L L L L L L + +	83	447	335
Pilule vaccine ... Sample 2	1 pilule	2 pilules	L L L L L L L L L L +	92	402	321
Pilule vaccine ... Sample 2	½ pilule	1 pilule	L L L L L L L L L L + +	83	424	365
Controls	—	—	+ + + + + + + + + + + + + + + +	0	406	348

+ = Death, confirmed due to *Cl. chauvoei* infection, within five days of infection. L = Lived.

work, recorded earlier, was utilised in subsequent tests; secondly, it was evident that such a vaccine could be prepared only from a broth culture which could be depended upon to be highly infective and to yield constant results when treated with ammonium sulphate. A considerable amount of work was carried out on the latter aspect of the problem, but as this was related to the growth of many organisms other than *Cl. chauvoei*, only brief details need be quoted. The most outstanding difficulty met with in using broth media, especially those most favourable to the growth of *Cl. chauvoei*, was their tendency to yield an excessive precipitate with ammonium sulphate, so that the amount which could be incorporated in a pilule corresponded to a very small amount of parent culture. As the quantity of precipitate obtained was a factor of the amount of undenatured or undigested protein in the medium, we considered the possibility of growing the organism in broth consisting of a watery solution of a protein-free peptone prepared at the Institute. It was found that abundant growth and adequate infectivity could be obtained in a 4 per cent. solution of this peptone in saline with 0·5 per cent. of glucose; this culture also gave a sufficiently small precipitate with ammonium sulphate. It was later shown that an improvement could be made in infectivity and in weight of growth by adding sterile normal horse serum to the medium, and further that it was then possible to reduce the amount of peptone to 2 per cent. As serum adds considerably to the amount of precipitable material in the final culture, it was necessary to determine the minimum amount which would increase growth; this was found to be 0·1 per cent.

There are therefore two media which give satisfactory results, composed as follows :—

(1)		(2)	
Peptone (Evans)	4 g.	Peptone (Evans)	2 g.
Sodium chloride	0·5 g.	Sodium chloride	0·5 g.
Glucose ...	0·5 g.	Glucose	0·3 g.
Distilled water to	100 ml.	Normal horse serum (total solids adjusted to 6·5 per cent.)	0·1 g.
		Distilled water to... ...	100 ml.

The water, peptone and salt are heated to 90° C. to precipitate phosphates; the medium is then filtered, autoclaved, cooled to 37° C. and the serum and glucose added.

Sample 2 in Table IV was prepared from the first medium and Sample 1 from the second medium.

Preparation of the Vaccine.—The strain of *Cl. chauvoei* is maintained in desiccated muscle. The broth is inoculated with one tube of culture in meat broth medium, and incubated at 37° C. for 48 hours. A sample is removed for virulence tests in guinea-pigs and 0·4 per cent. of formalin added to the remainder. It is then incubated for a further 14 days, and left at room temperature for 14 days. Its

toxicity is ascertained by subcutaneous injection into the abdominal wall of each of five guinea-pigs. If the animals survive and show no necrosis at the site of inoculation, the preparation of the vaccine is proceeded with. The organisms are removed by syphon and centrifuge and are stored in the form of a thin cream. Ammonium sulphate in the proportion of 700 grammes to each litre is added to the supernatant liquid and the resulting precipitate is dried. The weight of the dried precipitate and the volume of the bacterial suspension having been ascertained they are combined in the pilule in the proportion in which they were present in the parent culture. At the time of preparation 85 per cent. of the pilule consists of the ammonium sulphate precipitate and 10 per cent. of the bacterial suspension, the remaining 5 per cent. being excipient.

As it is possible to include sufficient material in the new pilules only by exceeding the size of those now in use, the " pilules " were prepared in the form of short cylinders, with a diameter adjusted to fit the injecting apparatus in common use. There is no reason to believe that this form would be less convenient to use in the field than the present spherical one. The pilules used in these experiments corresponded to 2·5 ml. of parent culture and as many as three can be implanted subcutaneously in one operation.

TESTS FOR ANTIGENICITY

The vaccine was submitted to the test described above and at the same time tests were carried out on the parent formalin killed cultures. The results of two such experiments recorded in Table IV show that the pilule vaccines prepared from culture conferred a substantial degree of immunity upon guinea-pigs and compared favourably with formalinised culture. Even when the dose of pilule vaccine is reduced to correspond to half of the amount of formalinised culture (Experiment 2, Table IV) the number of guinea-pigs protected was equal in the two groups. When the results of the two experiments are combined it is found that formalinised culture protected 77·3 per cent., the cultural pilules protected 87·9 per cent., while none of the control guinea-pigs survived.

During the course of Experiment 2 there was a fall in the weights of the guinea-pigs, due to feeding difficulties. In the guinea-pigs protected with the dry vaccine, this fall is comparable with that in the control group, but is somewhat greater in the group protected with formalinised culture. This accords with the impression gained in both experiments that dry vaccine was at least as innocuous to guinea-pigs as the liquid vaccine.

DISCUSSION

The evidence obtained in this work seems to indicate that blackquarter prophylaxis could be brought into a satisfactory state with little alteration in current practise. There appears to be some justification

for a recommendation that the principal agent should be formalinised whole culture, with culture pellet vaccine available for practitioners who have a strong preference for this type of preparation. The results shown in Table II are in themselves strong reasons for abandoning other forms of vaccination which also possess added disadvantages. Thus, the muscle powder pellets are prepared by a cumbersome method which cannot be standardised; indeed, in our experience its preparation, in accordance with the original principles, is a procedure requiring considerable skill. If it is claimed that the immunity it produces in cattle cannot be demonstrated in guinea-pigs, then it has the disadvantage that it cannot be tested for potency; in addition, the presence of living spore-bearing organisms in the vaccine makes it an undesirable agent to handle side by side with sterile biological materials. Finally, its preparation entails the slaughter of healthy cattle. We have been able to demonstrate that "Natural Aggressin" can be prepared in such a manner as to constitute a satisfactory vaccine, but its preparation entails filtration troubles which would be difficult to overcome in large-scale production, and also necessitates the slaughter of normal animals. Artificial filtrate or culture filtrate has few undesirable features but is of course wholly condemned by the results shown in Table II. In view of these results, which show that manufacturers who market satisfactory formalinised whole culture do not always prepare satisfactory filtrate, it would seem unwise to encourage the use of the latter.

If this recommendation is accepted, then the test described above should not set too high a standard for potency as the majority of commercial samples of formalinised culture satisfied the test. Opportunities for the testing of the new pellet vaccine described above on the same scale have not occurred, but from our limited experience with it there seems to be no reason to suppose that it would not satisfy the test.

SUMMARY

A test, based on the immunisation of guinea-pigs, and designed to determine whether a blackquarter vaccine is a satisfactory antigen, is described.

A survey of 20 samples of blackleg vaccines, mainly purchased in the open market, has been carried out. It was found that eleven of these had little or no immunising value, and that seven of the nine agents which satisfied the test were formalin killed cultures. Pellet vaccines prepared from attenuated infected muscle, appeared to have no immunising value.

A new form of pellet vaccine, designed for the needs of practitioners who prefer this method of administration, is described.

The opinion is expressed that immunising agents for blackquarter should be limited to two products, namely, formalin killed whole culture and culture pellets, and that these should be submitted to the test outlined.

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EXPERIMENTAL *STAPHYLOCOCCUS AUREUS* MASTITIS IN SHEEP
TREATMENT TRIAL WITH SULPHONAMIDES OR STAPHYLOCOCCUS TOXOID

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INTRODUCTION

Acute staphylococcal mastitis in ewes occurring in Great Britain and many other countries has been fully reviewed by Minett (1939). Most of the reports deal with aetiology or prevention and nothing appears to have been published regarding treatment. In some recent outbreaks in this country, claims have been made (unpublished) that sulphonamide drugs, particularly sulphapyridine, are of curative value. It was decided therefore to make this subject the basis of an experiment.

The two drugs selected were sulphapyridine and sulphathiazole, the former on account of its reputed value in the field and the latter because it is known to be more active on *Staphylococcus aureus* than sulphapyridine or any of the other sulphonamides at present available; staphylococcus toxoid was also studied because its use therapeutically as well as prophylactically has been suggested. Gould (1942) and Wilson (1942) found it of value in subacute cases of staphylococcal mastitis in cows.

The strain of staphylococcus (P) used in the present investigation was isolated in pure culture from the udder secretion of a field case of ovine mastitis.

TITRATION EXPERIMENT

A preliminary titration of the pathogenicity of the staphylococcus culture was made in nine lactating sheep. The organism was grown for 24 hours in serum broth at 37° C. and ten-fold dilutions made in peptone broth. The sheep were divided into three groups and were inoculated into one half of the udder by the teat orifice with the culture and its dilutions as follows:—

Group 1. 1 c.c. whole culture—approx. 500 million organisms per c.c.
„ 2. „ 1/10 dilution „ 50 „ „ „
„ 3. „ 1/100 dilution „ 5 „ „ „
(Viable counts were made by serum-agar pour-plates).

Within 24 hours, all six sheep in Groups 1 and 2 developed severe mastitis which progressed towards necrosis and sloughing of part of the udder at the end of twelve days. In the third group

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reactions were mild; in two cases the milk remained normal in appearance throughout the experiment but there was slight swelling and induration of the udder in one animal. The third ewe developed mastitis of moderate severity but when destroyed twelve days after inoculation there was no necrosis of the udder.

It was therefore decided to employ the inoculum used in Group 2, *i.e.*, 1 c.c. of a 1 in 10 dilution of a 24 hour serum broth culture, in the subsequent experiment.

TREATMENT TRIAL

Method.—Thirty-six ewes of mixed breeds with lambs were used. The lambs were removed at the beginning of the experiment and the ewes' udders were examined. Samples of milk from all the ewes, collected with precautions against contamination, were examined bacteriologically on two occasions on successive days. As the ewes were going dry, it was not possible to complete the examination of the pre-inoculation samples of milk before proceeding with the main experiment. On the day of inoculation the ewes were divided into four groups of nine. One group (controls) was kept in a separate building; the three other groups were housed in one large shed, divided into three sections by hurdles.

Inoculation.—A 24 hour serum-broth culture of *Staphylococcus aureus,* strain P, which had been kept in meat broth since isolation, was diluted ten times in peptone broth. An inoculation of 1 c.c. of this dilution containing 14,300,000 viable organisms (counted by serum-agar pour-plates), was made by way of the teat orifice, using a blunt cannula attached to a 1 c.c. syringe. When available the left half of the udder was inoculated, but in sheep Nos. 701, 688 and 734 the right halves were used since the left halves were completely atrophied.

It was decided to begin treatment at about the time that a diagnosis of mastitis could have been made in practice and the sheep were therefore examined every six hours. Blood samples were collected from the control sheep at the sixth, 12th, 18th, 24th, and 48th hours for bacteriological examination, to determine whether or not there was a bacteraemic phase of the infection.

Between six and seven hours after inoculation the sheep were breathing rapidly and had an anxious appearance. The temperatures of the control group ranged from 106·5° to 108·4° F. with an average of 107·3° F. The udders, however were normal in appearance and treatment was withheld for the time being. When next examined between the 12th and 13th hours, many of the sheep were lame and in all groups most of the udders were hot, swollen and painful on the inoculated side. The milk in most cases was either little altered or slightly tinged with blood. A few had already passed to a stage of coldness of the udder and discolouration of the skin, with raised ridges (weals) on the affected halves, usually round about the teat; the milk was distinctly red in colour. Three (Nos. 749, 692 and 738) still showed no abnormality of the udder, although they were in a state of fever.

Treatment

Treatment was begun at the 13th hour after inoculation and the three groups were dealt with in the order: (1) Sulphapyridine, (2) Sulphathiazole, (3) Toxoid.

In view of the relative insusceptibility of staphylococci to sulphonamides it was decided to employ sulphapyridine and sulphathiazole in as high a dosage as possible. The sheep weighed from approximately 110 to 125 lb. The drugs were suspended in gum tragacanth, and the sheep were dosed by mouth with a dosing syringe.

Sulphapyridine.—Treatment with sulphapyridine was commenced at 12 midnight on June 12th with doses of 12 g. per head. Thereafter, 6 g. were given at 12-hourly intervals, beginning at 9.30 a.m. on June 13th until the morning of June 16th, when treatment was stopped. This represented an initial dose of approximately 1 g. per 10 lb. body weight followed by a 24 hourly intake of the same amount for four days.

Sulphathiazole.—The sheep of the sulphathiazole group were given 12 g. as the initial dose followed by two doses of 6 g. at intervals of six hours each (6.30 a.m. and 12.30 p.m. on June 13th). A third dose of 6 g. was given at 9.30 p.m. and thereafter at intervals of 12 hours until the morning of June 16th. This represents an initial dose of 1 g. per 10 lb. followed by a daily average of approximately 1g. per 7lb. divided into two or three administrations over a period of four days.

Toxoid.—The staphylococcus toxoid used was batch No. W.4565B prepared by the Wellcome Physiological Research Laboratories. The first dose consisting of 5 c.c. was injected intramuscularly shortly after midnight on June 12th. The second, of 10 c.c., was given at 9.30 p.m. on June 14th, *i.e.*, two days later.

Clinical Course of the Disease

At 8 a.m. on June 13th (21 hours after inoculation) most udders were extremely hard, swollen and cold, becoming blue in colour. The secretion was red and watery in character. Two days later, the colour of the udder gradually changed to a pallid grey and serum began to ooze through the skin. Two sheep in the sulphathiazole group and one in the sulphapyridine group died on the third day. Sulphonamide poisoning was suspected. From the third day onwards, those most severely affected had gas in the affected halves; in addition, an oedematous swelling of the subcutaneous tissues extended from the udder along the lower surface of the abdomen. After 14 days there was complete necrosis of the injected half of the udder and sloughing of the overlying skin. In the toxoid group one sheep died on each of the sixth, seventh and eighth days and one in the control group on the 14th day. After the stage of pain and fever had passed, the surviving sheep ate a certain amount of food and drank a good deal of water; they also became brighter in appearance although at

the end of 14 days they were still in very poor condition and had lost weight.

As regards the separate groups of sheep there appeared to be little difference between the three treated groups and the controls, but as will be seen from the table there was considerable variation in the severity of the symptoms in individual animals.

Milk samples for bacteriological examination were collected from both halves of the udders on the second day after inoculation, and again, from those which had not reached the stage of complete necrosis, on the 14th day.

Post-mortem findings.— In each sheep which died after the sixth day the udder showed a dark red gelatinous oedema of the subcutaneous tissue which extended forward on to the abdominal floor. In one case there was necrosis of the internal muscles of the inguinal area and the thigh. The glandular tissue of the udder had a dull appearance but there were no visible lesions.

Bacteriological Examination of Blood and Milk Samples

(a) *Blood.*—Blood samples were prevented from clotting and leucocyte action inhibited by the addition of "Liquoid"* at the time of collection. Immediately after collection 0·1 c.c. was spread on the surface of blood agar plates and incubated for 48 hours at 37° C. Examination revealed haemolytic colonies of *Staphylococcus aureus* in very small numbers (one colony, seven colonies, one colony) on three plates only. They were derived from samples from three different sheep, collected at 18 hours, 18 hours and 48 hours respectively. As the plates in question also contained a number of non-haemolytic staphylococci and other skin contaminants, the finding was not regarded as implying a bacteraemia.

(b) Milk.—The milk samples collected before and after inoculation were examined by spreading a loopful of milk on the surface of a blood agar plate which was incubated for 48 hours. Any β-haemolytic colonies of staphylococci or streptococci were picked off into serum broth for further examination. Only the samples from the inoculated halves of the udders are shown in the table.

Results

Examination of milk samples taken before inoculation.—The milk from the left half of the udder of No. 711 was thick and greenish yellow in colour and contained haemolytic staphylococci. Haemolytic staphylococci were also found, in the halves of the udders subsequently inoculated, in sheep Nos. 668 and 707. Nos. 9966 and 693 were excreting coliform organisms from the right (uninoculated) halves and this was associated with the presence of clots in the milk: in one case there was hardness and enlargement of the affected half. Two of the sheep (Nos. 749 and 744) were already dry and the udders contained only whey-like material, while No. 9985 was almost dry. The remainder were normal.

* Roche Products Ltd.

Examination of milk samples after inoculation. Haemolytic staphylococci were isolated in varying but usually large numbers from all inoculated halves of the udders on both the second and 14th days after inoculation. Samples of secretion from udders containing gas, cultured under anaerobic conditions, contained haemolytic staphylococci, but no other organisms.

Examination of Films

Films of the infected milk stained by Leishman showed large numbers of leucocytes, mostly polymorphonuclears, many of which were damaged and dead. A few contained engulfed cocci, but in most cases there were large masses of free staphylococci.

Microscopical Examination of Udder Sections

Sections from the udders of sheep which died during the experiment revealed interlobular oedema and leucocytic infiltration as well as extensive subcutaneous oedema. In some areas small abscesses had formed while the milk alveoli contained blood and bacteria.

Estimations of Sulpronamide Concentrations

It was not possible to carry out a large number of estimations, but blood samples were collected at random from three sheep in each of the two drug groups immediately after dosing had been completed on the morning of June 15th. Although the levels at this time would have risen slightly above their minimum owing to the rapid absorption of both drugs it was expected that a rough indication of the approximate blood levels in the two groups during treatment would be obtained.

Estimations of the concentration in the milk were also carried out on two samples from each of the two drug groups collected from the uninoculated halves of the udders at 2 p.m. on June 14th (the second day of treatment—approximately 4½ hours after the last dose of drug).

	Sheep No.	*Time*	*Mgm. per* 100 *cc. (free drug)*
Sulphapyridine:			
Blood	688	15/6 10 a.m.	13·2
	737	„ „	13·2
	690	„ „	14·4
Milk	690	14/6 2 p.m.	9·6
	711	„ „	10·8
Sulphathiazole:			
Blood	9954	15/6 10 a.m.	5·4
	722	„ „	4·8
	9966	„ „	7·2
	9987	14/6 9 p.m.	20·4
Milk	9960	14/6 2 p.m.	7·2
	718	„ „	2·4

Sheep No.	Milk samples: Before inoculation — Appearance	Bacteriology	48 hours after inoculation — Appearance	HS infection	14 days after inoculation — Appearance	HS infection	Clinical description of udders on examination at the 12th, 21st, 34th and 51st hours and 3rd and 14th days
Control Group							
745	N	—	Brown, watery	+++	US		Swollen, hard, 12th hour. Cold, blue, 34th hour. Necrosis and sloughing, 14th day
701	N	—	Reddish	+++	US		Swollen, hot, till 3rd day then cold, blue. Necrosis and sloughing, 14th day
689	Whey	—	Yellow-brown	+++	Brown, thick	+++	Slightly swollen, warm, hard throughout 14 days
749	N	—	Greenish	+++	Green, purulent	+++	Swollen, hard, warm, at 51st hour and on 3rd day. Normal on 14th day
723	N	—	Brown, watery	+++	US		Swollen, 12th hour. Cold, blue, 21st hour. Oedema, 51st hour. Sloughing 14th day
731	N	—	„	+++	D		Swollen, cold, blue, 12th hour. Gas and oedema, 51st hour. Died 14th day
740	N	—	„	+++	US		Swollen, cold, blue, 21st hour. Gas and oedema, 51st hour. Necrosis and sloughing, 14th day
742	N	—	„	+++	US		„ „ „ „
744	Whey	Few NHS	Yellow-brown	+++	Reddish curd	+++	Swollen, hot, 21st hour. Blue weal, 51st hour. Small area necrosis, 14th day
Sulphapyridine Group							
707	N	HS	N	++	Buff, granular	+	Slightly swollen, hard, 12th hour. Normal, 3rd and 14th days.
735	N	—	Brown and clots	++++	US		Swollen, cold, 12th hour. Gas, 34th hour. Necrosis and sloughing, 14th day
698	N	—	N	++	Yellow, granular	+++	Remained normal throughout
737	N	—	Brown and clots	+++	US		Swollen, hard, cold, 21st hour. Gas and oedema, 51st hour. Sloughing, 14th day
746	N	—	Brown, watery	++++	D		Swollen, hard, blue, cold, 21st hour. Sheep moribund, 51st hour. Died on 3rd day
690	N	—	„	+++	US		Swollen, cold, blue, 21st hour. Gas and oedema, 3rd day. Sloughing, 14th day
688	Clots	HS	Clear and clots	++	Clear and clots	+	Slightly swollen, hard, till 3rd day. Normal, 14th day

711	Abn.*	Few HS	Greenish	+++	"	+	
747	N	—	Brown, watery	+++	US		Swollen," cold, blue, 21st hour." Gas at 51st hour." Sloughing on 14th day
Sulphathiazole Group							
9954	N	Few NHS	Greenish	++	Reddish curd	+++	Hard, hot, 12th and 21st hour. Slightly swollen, 3rd day. Normal, 14th day
9966	N	—	Brown, watery	++++	US		Swollen, cold, 12th hour. Gas and oedema, 51st hour. Necrosis and sloughing, 14th day
9960	N	—	Yellow-brown	+++	D		Swollen, hard, hot, 12th to 51st hour. Died on 3rd day
9987	N	—	"	++++	D		
9985	Thin	—	N	+	Buff, granular	+++	Slightly" swollen, 12th hour. Normal, 21st hour and after.
693	N	—	Dirty yellow	+++	Reddish curd	+++	Swollen, hard, hot, 12th hour and throughout 14 days
718	N	—	Brown, watery	+++	US		Swollen, cold, 21st hour. Blue, 34th hour. Oedema, 3rd day. Sloughing, 14th day
692	N	H. strept.	Yellow	++	Clear and clots	+	Remained normal throughout
722	N	—	Brown, watery	+++	US		Swollen, hard, cold, 21st hour. Blue and gas at 51st hour. Sloughing, 14th day
Toxoid Group							
684	N	—	Brown, watery	+++	US		Swollen, hard, 12th hour. Cold, 34th hour. Blue, oozing, 3rd day. Sloughing, 14th day
—	N	—	Greenish	+++	Yellow curd	+++	Swollen, hard, hot, 12th hour and throughout 14 days
734	N	—	Brown, watery	+++	US		Swollen, cold, blue, 12th hour. Gas, 51st hour. Necrosis and sloughing, 14th day
720	N	—	Brown and clots	+++	D		Swollen, cold, blue, 21st hour. Gas, 34th hour. Oedema, 3rd day. Died, 6th day
738	N	—	Greenish	+++	Reddish-brown	+++	At first normal. Swollen, hard, hot, 3rd and 14th days
741	N	—	Brown, watery	+++	D		Swollen, cold, blue, 21st hour. Gas and oedema, 3rd day. Died, 8th day
730	N	—	"	+++	US		Swollen, hard, 21st hour. Cold, blue, 51st hour. Sloughing, 14th day
712	N	—	"	+++	D		Swollen, cold, blue, 21st hour. Gas, 51st hour. Died, 7th day.
703	N	—	"	+++	US		Swollen, hard, 12th hour. Cold, blue and oedema, 3rd day. Sloughing, 14th day

*Abnormal = Thick greenish yellow. N = Normal. NHS = Non-haemolytic staphylococci. HS = Haemolytic staphylococci.
H. strept. = Haemolytic streptococci. US = Udder sloughing. D = Dead.

DISCUSSION

From a consideration of our own results and those of Minett and others it seems certain that there are several factors which play a part in the pathogenesis of the disease, *viz.*, the weight of infection, the stage of lactation, the existence of a previous immunity, and, probably, enviromental factors such as cold and wet.

Although the infection produced in our experiments was severe, the number of organisms inoculated does not appear to have been excessive as it consisted of only three times that which failed to cause clinical mastitis in two out of three sheep in the titration experiment. Moreover it was much lower than that used by Minett (1939). An important difference between Minett's experiments and ours, however, may have been in the stage of lactation of the ewes. We used animals which were either in milk or just drying off, whilst nearly all of his had long passed their lactation and were completely dry. In the one case in which he used a lactating ewe he records an unusually severe infection. Amongst our ewes, moreover, there appeared to be a definite correlation between the severity of the symptoms and the stage of lactation. Minett also found considerable variations, sometimes amounting to several units, in anti-toxin content of the blood of sheep and concluded that this factor influenced the severity of infection. We did not examine our ewes in this way but we consider that the variability of the infection in different sheep may also have been connected with the anti-toxin content of the blood. It may be noted that three sheep, Nos. 707, 688, and 711, which were already infected with haemolytic staphylococci in the udder before inoculation, were amongst those least severely affected.

It is clear that none of the methods of treatment was of any value under the conditions of the experiment. Although the sulphapyridine concentrations obtained in the blood and milk indicated that dosage with this drug was fully adequate and could not have been increased with safety, necrosis of the udder occurred in four cases and staphylococci were not eliminated from any of the sheep treated. sulphathiazole concentrations in the samples examined were lower than those of sulphapyridine due no doubt to the more rapid excretion of the former drug. It might be expected, however, that somewhat higher concentrations existed during the first 24 hours of treatment when more frequent administration was carried out. Nevertheless, treatment was of no value as staphylococci were not eliminated and necrosis and sloughing of the udders took place in three sheep.

Owing to pressure of work it was not possible to confirm fully that sulphonamide poisoning was the cause of death in sheep Nos. 9960, 9987 and 746, but the very high blood concentration of sulphathiazole found in No. 9987 shortly before death is suggestive of excretory failure. In addition it is significant that all three deaths

took place relatively early in the course of the disease (at least three days before any deaths occurred in other groups).

It hardly seems reasonable to expect that toxoid used therapeutically could be of immediate value in a disease so acute and rapidly progressing. It is possible that, if toxoid treatment had been continued in the sheep whose udders were not necrotic at the end of the experiment, the staphylococci might eventually have been eliminated. Toxoid did not, however, prevent death in three cases nor prevent the development of necrosis of the udder in four others.

SUMMARY

The inoculation of a 24 hour, serum broth culture of *Staphylococcus aureus* containing 500 million organisms per c.c. into the udders of three lactating ewes *via* the teat canal set up an acute mastitis. A similar result was obtained with one-tenth of the dose, but one-hundredth of it produced only a mild reaction in two ewes and a moderate infection in the third.

Thirty-six ewes in late lactation developed mastitis in one half of the udder by the inoculation of approximately 14 million haemolytic staphylococci. The infection was severe in the majority of sheep but there was considerable individual variation amongst the remainder.

Cultural examination of the blood at six, 12, 18, 24 and 48 hours after inoculation failed to demonstrate a bacteraemic phase.

Treatment of three groups of nine affected ewes with sulphathiazole, sulphapyridine and staphylococcus toxoid respectively proved ineffective. In spite of the initiation of treatment as soon as the first symptoms of mastitis were detected (13 hours after inoculation), the disease progressed, and staphylococci were still present in the udders 14 days after inoculation.

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* *Meyer, E., and Arnold, I., Amer. J. Digest. Dis., 5:418*

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